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(54) Title: METHODS FOR CLEAVING DNA WITH NUCLEOTIDE INTEGRASES (57) Abstract The present invention provides methods, using a nucleotide integrase, for cleaving nucleic acids substrates at specific sites and inserting a nucleic acid molecule into the cleaved substrate. The method of cleaving one strand of a double-stranded DNA substrate comprises providing a nucleotide integrase comprising a group II-intron RNA having two hybridizing sequences capable of hybridizing with two intron RNA binding sequences on the one strand of the substrate and a group II-intron encoded protein which binds to a first sequence element of the substrate. The method of cleaving both strands of a double-stranded DNA substrate comprises providing a nucleotide integrase comprising a group II-intron RNA having two hybridizing sequences capable of hybridizing with two intron RNA binding sequences on one strand of the substrate and a group II-intron encoded protein capable of binding to first and second sequence elements in the recognition site of the substrate. The method of cleaving a single-stranded nucleic acid substrate comprises providing an integrase having two hybridizing sequences capable of hybridizing with two intron RNA-binding sequences of the substrate and a group II-intron encoded protein.		

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METHODS FOR CLEAVING DNA WITH NUCLEOTIDE INTEGRASES

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BACKGROUND

In recent years, a number of methods have been developed for manipulating DNA. Some of these methods employ biomolecules to cut or cleave DNA, which in some instances renders the substrate DNA nonfunctional. Other methods employ biomolecules to facilitate insertion of new pieces of nucleic acid into the cleavage site of the DNA substrate. The insertion of new segments of nucleic acid into the cleavage sites of the DNA substrate changes the characteristics of the RNA or protein molecules encoded by the substrate DNA molecules. Accordingly, the biomolecules which catalyze the cleavage of DNA substrates or the insertion of new nucleic acid molecules into the DNA substrates are useful tools for genetic engineering, for analytical studies and for diagnostic studies. One such molecule used for cleaving DNA substrates is the restriction endonuclease.

Restriction endonucleases are enzymatic proteins that cleave double-stranded DNA. Such endonucleases recognize specific nucleotide sequences in double-stranded DNA, and cleave both strands within or near the specific recognition site. Such specificity renders the restriction endonucleases important tools in the controlled fragmentation of double-stranded DNA. Restriction endonucleases are also useful analytical tools for determining whether certain sequences are present in substrate DNA and in genomic sequencing studies.

However, restriction endonucleases only cleave DNA substrates; they do not insert new nucleic acid molecules into the cleaved DNA substrate. Accordingly, another biomolecule is needed to insert new pieces of DNA or RNA into the double-stranded DNA.

Ribozymes are catalytic RNA molecules that cleave RNA and, in certain circumstances, that insert new pieces of RNA into the cleavage site of the RNA substrate. Unfortunately, ribozymes have not been particularly useful for cleaving single-stranded DNA substrates or double-stranded DNA substrates. Ribozymes cut single-stranded DNA only under extreme conditions of elevated temperatures and high concentrations of magnesium. Ribozymes can be used to cleave double-stranded DNA only after the DNA is denatured and separated into two pieces of single-stranded DNA. Moreover, ribozymes have limited use in systems containing ribonucleases.

Accordingly, it is desirable to have new methods that employ a new tool that is capable of cleaving double-stranded DNA molecules, single-stranded DNA molecules, and single-stranded RNA molecules at specific sites. Methods which employ a new biomolecule capable of cleaving RNA molecules, single-stranded DNA molecules and double-stranded DNA molecules at specific sites and simultaneously inserting a new nucleic acid molecule into the cleavage site are especially desirable.

SUMMARY OF THE INVENTION

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The present invention provides new methods, employing a nucleotide integrase, for cleaving single-stranded RNA substrates, single-stranded DNA substrates, and double-stranded DNA substrates at specific sites and for inserting nucleic acid molecules into the cleaved substrate. The nucleotide integrase is a ribonucleoprotein particle comprising a group II intron RNA and a group II intron-encoded protein, which is bound to the group II intron RNA.

One method uses a nucleotide integrase to cleave one strand, hereinafter referred to as the "top strand" of a double stranded DNA substrate. As denoted herein, nucleotides that are located upstream of the cleavage site on the top strand have a (-) position relative to the cleavage site, and nucleotides that are located downstream of the cleavage site have a (+) position relative to the cleavage site. Thus, the cleavage site is located between nucleotides
5 -1 and +1 on the top strand of the double-stranded DNA substrate. The top strand of the substrate comprises a first intron RNA binding sequence, hereinafter referred to as the "IBS1" sequence and a second intron RNA binding sequence, hereinafter referred to as the "IBS2" sequence. The IBS1 sequence and IBS2 sequence lie in a region which extends from about position -1 to about position -14 relative to the cleavage site. The first 10 to 12 pairs of nucleotides that lie upstream of IBS2 and IBS1, i.e from about position -12 relative to the cleavage site to about
10 position -24 relative to the cleavage site are hereinafter collectively referred to as "the first sequence element". The first 10 to 12 pairs of nucleotide that lie downstream of the cleavage site are hereinafter collectively referred to as "the second sequence element".

The method comprises the steps of: providing a nucleotide integrase comprising a group II intron RNA having two hybridizing sequences, "EBS1" and "EBS2", that are capable of hybridizing with the IBS1 sequence and
15 IBS2 sequence, respectively, on the top strand of the DNA substrate, and a group II-intron encoded protein which binds to at least one nucleotide in the first sequence element of the substrate; and reacting the nucleotide integrase with the double-stranded DNA substrate under conditions that permit the nucleotide integrase to cleave the top strand of the DNA substrate and to insert the group II intron RNA into the cleavage site. Preferably, the nucleotide immediately
20 preceding the first nucleotide of the EBS1 sequence on the group II intron RNA, hereinafter referred to as the δ' nucleotide is complementary to the nucleotide at +1 on the top strand of the substrate, hereinafter referred to as the δ' nucleotide. The EBS1 sequence of the group II intron RNA comprises from about 5 to 7 nucleotides and has substantial complementarity with the nucleotides at positions -1 to about -5 or about -7 on the top strand of the DNA substrate. The EBS2 sequence comprises from about 4 to 7 nucleotides and has substantial complementarity with the nucleotides at positions from about -6 to about -14 on the top strand of the DNA substrate.

25 The present invention also provides a method which employs a nucleotide integrase to cleave both strands of a double-stranded DNA substrate. The method comprises the steps of: providing a nucleotide integrase comprising a group II intron RNA having two hybridizing sequences, EBS1 and EBS2, that are capable of hybridizing with two intron RNA binding sequences, IBS1 and IBS2, on the top strand of the substrate, and a group II-intron encoded protein that is capable of binding to at least one nucleotide in the first sequence element and to at least one
30 nucleotide in a second sequence element in the recognition site of the substrate; and reacting the nucleotide integrase with the double-stranded DNA substrate such that the nucleotide integrase cleaves both strands of the DNA substrate and inserts the group II intron RNA into the cleavage site of the top strand. Preferably, the δ nucleotide of the group II intron RNA is complementary to the δ' nucleotide on the top strand of the substrate.

Another method provided by the present invention employs a nucleotide integrase for cleaving a
35 single-stranded nucleic acid substrate and for inserting the group II intron RNA of the nucleotide integrase into the cleavage site. The method comprises the steps of: providing a nucleotide integrase having two hybridizing sequences, EBS1 and EBS2, that are capable of hybridizing with two intron RNA-binding sequences, IBS1 and IBS2, on the

single-stranded substrate, and a group II intron encoded protein; and reacting the nucleotide integrase with the single stranded nucleic acid substrate for a time and at a temperature sufficient to allow the nucleotide integrase to cleave the substrate and to attach the group II intron RNA molecule thereto. The EBS1 sequence of the group II intron RNA comprises from about 5 to 7 nucleotides that have substantial complementarity with the nucleotides at positions -1 to about -5 or about -7 relative to the putative cleavage site. The EBS2 sequence comprises from about 4 to 7 nucleotides that have substantial complementarity with the nucleotides at positions from about -6 to about -14 relative to the putative cleavage site. Preferably, the 8 nucleotide of the group II intron RNA is complementary to the 8' nucleotide on the top strand of the substrate.

The present invention also relates to a method of determining whether a nucleic acid comprises a particular recognition site. The method comprises the steps of providing a nucleotide integrase capable of cleaving a nucleic acid comprising a particular recognition site; reacting the nucleotide integrase with the nucleic acid; and assaying for cleavage of the nucleic acid, wherein cleavage of the nucleic acid indicates that the nucleic acid comprises the recognition site.

BRIEF DESCRIPTION OF THE DRAWING

Figure 1 is a diagram of showing the interaction between the EBS sequences of a group II intron RNA of the second intron of the *S. cerevisiae* mitochondrial *COX1* gene, hereinafter referred to as the "aI2 intron" RNA and the IBS sequences of a DNA substrate. The cleavage site in the substrate is represented by an arrow.

Figure 2 is a diagram depicting the nucleotide sequence and the of the aI2 intron RNA, SEQ.ID.NO.1 and the nucleotide sequence of the group II intron RNA of the first intron of the *S. cerevisiae* mitochondrial *COX1* gene, hereinafter referred to as the "aI1 intron" RNA, SEQ.ID.NO.2. Markings above the sequence identify the position of the EBS1 sequence and the EBS2 sequence of the wild-type aI1 intron RNA and the wild-type aI2 intron RNA.

Figure 3 is a chart depicting the sequence of a DNA substrate cleaved by a nucleotide integrase comprising a wild-type aI2 intron RNA and the protein encoded thereby and the position of the point mutations made in this sequence.

Figure 4 is a graph showing the relative extent of cleavage of the substrates having mutations in the first sequence element by a nucleotide integrase comprising a wild-type aI2 intron RNA and the protein encoded thereby.

Figure 5 is a graph showing the relative extent of cleavage of the substrates having mutations in the second sequence element by a nucleotide integrase comprising a wild-type aI2 intron RNA and the protein encoded by the aI2 intron RNA.

Figure 6 is a chart depicting the sequence of a DNA substrate cleaved by a nucleotide integrase comprising a wild-type aI1 intron RNA, and the protein encoded by the aI1 intron RNA and the position of the mutations made in this sequence.

Figure 7 is a graph showing the relative extent of cleavage of the substrates having mutations upstream of the cleavage site by a nucleotide integrase comprising a wild-type a11 intron RNA and the protein encoded thereby.

Figure 8 is a chart depicting the sequence of a DNA substrate cleaved by a nucleotide integrase comprising a wild-type group II intron RNA of the *Lactococcus lactis* *ltrB* gene, hereinafter referred to as the "L1.*ltrB* intron" RNA, and the protein encoded thereby, hereinafter referred to as the *ltrA* protein.

Figure 9 is a graph showing the relative extent of cleavage of the substrates having mutations in the first sequence element by a nucleotide integrase comprising a wild-type L1.*ltrB* intron RNA and the *ltrA* protein.

Figure 10 shows the L1.*ltrB* intron DNA sequence and portions of the nucleotide sequence of the flanking exons *ltrBE1* and *ltrBE2*, SEQ.ID.NO.5, the nucleotide sequence of the open reading frame, of the L1.*ltrB* intron SEQ. ID. NO. 6 and the amino acid sequence of the *ltrA* protein, SEQ.ID.NO. 7.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides new methods that employ a nucleotide integrase for manipulating DNA and RNA substrates.

One method uses a nucleotide integrase to cleave one strand, hereinafter referred to as the top strand, of a double-stranded DNA at a specific site and to concomitantly attach a nucleic acid molecule, which comprises an RNA molecule, to the cleaved strand at the cleavage site. The DNA substrate has a recognition site which comprises a first intron RNA binding sequence (IBS1) that is located on the top strand of the substrate and upstream of the cleavage site and a second intron RNA binding sequence (IBS2) that is located on the top strand of the DNA substrate and upstream of the IBS1 sequence. The recognition site also comprises a first sequence element that is located upstream of the IBS2 sequence. The first sequence element comprises from about 10 to 12 pairs of nucleotides.

The method of cleaving the top strand of a double-stranded DNA substrate comprises the steps of: providing a nucleotide integrase comprising a group II intron RNA having an EBS1 sequence and an EBS2 sequence that are capable of hybridizing with the IBS1 sequence and the IBS2, respectively on the top strand of the DNA substrate, and a group II-intron encoded protein capable of binding to at least one nucleotide in the first sequence element; and reacting the nucleotide integrase with the double-stranded DNA substrate for a time and at a temperature sufficient to permit the nucleotide integrase to cleave the top strand of the DNA substrate and to insert the group II intron RNA into the cleavage site. Preferably, the group II intron-encoded protein binds to a plurality of nucleotides in the first sequence element.

The nucleotide integrase employed in this method comprises a group II intron-encoded protein bound to an excised group II intron RNA. The EBS1 sequence and EBS2 sequence of the group II intron RNA have at least 80%, preferably 90%, more preferably full complementarity with the IBS1 sequence and IBS2 sequence, respectively, that are on the top strand of the substrate. The group II intron-encoded protein comprises an RT domain, an X domain, and the non-conserved portion of the Zn domain.

EBS1 is located in domain I of the group II intron RNA and comprises from about 5 to 7 nucleotides that are capable of hybridizing to the nucleotides of the IBS1 sequence of the substrate. EBS2 is located in domain I of

the group II intron RNA upstream of EBS1 and comprises from about 4 to 7 nucleotides that are capable of hybridizing to the nucleotides of IBS2 sequence of the substrate. If the nucleotides of the EBS1 and EBS2 sequences of the group II intron RNA are not at least 80% complementary to the nucleotides of the IBS1 or IBS2 sequences, respectively, then the group II intron RNA is modified to increase the complementarity between the EBS and IBS sequences. As shown in Fig. 1 the IBS1 sequence of the substrate is upstream of the cleavage site and the IBS2 sequence of the substrate is upstream of the IBS1 sequence.

In order to cleave the substrate efficiently, it is preferred that the nucleotide, δ , which immediately precedes the first nucleotide of EBS1 of the group II intron RNA, be complementary to the nucleotide at +1 in the top strand. Thus, if the δ nucleotide is not complementary to the nucleotide at +1 on the top strand of the substrate, the group II intron RNA is modified to contain a delta nucleotide which is complementary to the nucleotide at +1 on the top strand of the substrate. The nucleotide integrase is then reacted with the substrate. Suitable nucleotide integrases for use in this method include, for example the aI2 nucleotide integrase, the aI1 nucleotide integrase, and the ItrA nucleotide integrase.

The aI2 integrase comprises a wild-type or modified group II intron RNA of the second intron of the *S. cerevisiae* mitochondrial *COX1* gene, hereinafter referred to as the "aI2 intron" RNA, bound to a wild-type or modified aI2 intron encoded-protein. The sequence of the wild-type aI2 intron RNA is depicted in Fig. 1 and SEQ. ID. NO. 1. The sequence of the protein encoded by the wild-type aI2 intron RNA is set forth in SEQ. ID. NO. 3. EBS1 of the aI2 intron RNA comprises 6 nucleotides and is located at position 2985-2990 of the sequence set forth in SEQ. ID. NO. 1. EBS1 of the wild-type aI2 intron RNA has the sequence 5'-AGAAGA. The EBS2 sequence of the aI2 intron RNA comprises 6 nucleotides and is located at positions 2935-2940. The EBS2 sequence of the wild-type aI2 intron RNA has the sequence 5'-UCAUUA.

aI2 nucleotide integrases are used to cleave substrates that have on the top strand thereof a T at positions -15 and -13 relative to the putative cleavage site, a C at position -18 relative to the putative cleavage site, and a G at position -16 or position -19 relative to the putative cleavage site. Thus, to use the aI2 nucleotide integrase, one first examines the sequence of the top strand of the substrate to locate a target sequence 5'GCXXTXT or a target sequence 5'XCXGTX, wherein X represents A, C, G, or T and wherein A represents a nucleotide having an adenine base, G represents a nucleotide having a guanine base, C represents a nucleotide have a cytosine base, and T represents a nucleotide have a thymine base. Then, if the EBS2 sequence of the aI2 intron RNA does not have substantial complementarity to the IBS2 sequence, i.e., the sequence of 6 nucleotides that lie immediately downstream from one of these target sequences, and/or if EBS1 sequence of the aI2 intron RNA does not have substantial complementarity to the IBS1 sequence, i.e., the sequence of six nucleotides that lie immediately downstream of the IBS2 sequence, then EBS1 and EBS2 are modified to have substantial complementarity, as hereinafter explained. The efficiency of cleavage by the aI2 nucleotide integrase is increased if the top strand of the substrate has an A at -21, a G at -19, a C at -18, a G at -16, a T at -15, and a T at -13.

The aI1 nucleotide integrase comprises an excised, wild-type or modified excised group II intron RNA of the first intron of the *S. cerevisiae* mitochondrial *COX1* gene, hereinafter referred to as the "aI1 intron" RNA, and a wild-type or modified aI1 intron-encoded protein. The sequence of the aI1 intron RNA is shown in Fig.2 and

SEQ. ID. NO. 2. The sequence of the protein encoded by the a11 intron RNA is set forth in SEQ. ID. NO. 4. The EBS1 sequence of the a11 intron RNA comprises 6 nucleotides and is located at position 426-431. EBS1 of the wild-type a11 intron RNA has the sequence 5'-CGUUGA. The EBS2 sequence of the a11 intron RNA comprises 6 nucleotides and is located at positions 376-381. EBS2 of the wild-type a11 intron RNA and has the sequence 5'-ACAAUU.

a11 nucleotide integrases are used to cleave the top strand of double stranded DNA substrates that have on the top strand thereof a C at position -13 relative to the putative cleavage site. Preferably, the top strand of the substrate has a C at -13, a G at -22, a G at -21 an A at -19 and an A at -18 relative to the putative cleavage site. If the EBS2 sequence of the a11 intron RNA does not have substantial complementarity to the IBS2 sequence, i.e., the sequence of 6 nucleotides that lie immediately downstream from the C nucleotide at -13, and/or if EBS1 sequence of the a11 intron RNA does not have substantial complementarity to the IBS1 sequence, i.e., the sequence of six nucleotides that lie immediately downstream of the IBS2 sequence and immediately upstream of the cleavage site, then the EBS1 sequence and the EBS2 sequence of the group II intron RNA are modified to have substantial complementarity, as hereinafter explained.

The *ltrA* nucleotide integrase comprises an excised, wild-type or modified excised group LI.*ltrB* group II intron RNA of the *Lactococcus lactis* *ltrB* gene, hereinafter referred to as the "LI.*ltrB* intron" RNA, and a wild-type or modified LI.*ltrB* intron-encoded protein, hereinafter referred to as the *ltrA* protein. The sequence of the LI.*ltrB* intron RNA is shown in Fig. 10 and SEQ. ID. NO. 5. The sequence of the *ltrA* protein is set forth in SEQ. ID. NO. 7. The EBS1 sequence of the LI.*ltrB* intron RNA comprises 7 nucleotides and is located at positions 457 to 463. The EBS1 sequence of the wild-type LI.*ltrB* intron RNA has the sequence 5'-GUUGUGG. The EBS2 of the LI.*ltrB* intron RNA comprises 6 nucleotides and is located at positions 401 to and including 406. The EBS2 sequence of the wild-type LI.*ltrB* intron RNA has the Sequence 5'AUGUGU. The *ltrA* nucleotide integrase is used to cleave the top strand of a double-stranded DNA substrate when the top strand has a G at -21 and an A at -20 relative to the cleavage site. The *ltrA* nucleotide integrase cuts the top strand more efficiently when there is a G at -21, an A at -20, a T at -19, a G at -17, and a G at -15.

Another method uses a nucleotide integrase for cleaving both strands of double-stranded DNA and for attaching the group II intron RNA molecule into the cleavage site of the top strand of the DNA substrate. The nucleotide integrase comprises a group II intron-encoded protein bound to an excised group II intron RNA, wherein the group II intron RNA has an EBS1 sequence and an EBS2 sequence that have substantial complementarity to the IBS1 sequence and IBS2 sequence, respectively, on the top strand of the substrate. The EBS1 sequence comprises from about 5 to 7 nucleotides. The EBS2 sequence comprises from about 4 to 7 nucleotides. If the nucleotides of EBS1 and EBS2 of the group II intron RNA are not at least 80% complementary to the nucleotides of IBS1 and IBS2, the non-complementary nucleotides are modified, preferably, by recombinant techniques. Preferably, the δ nucleotide of the group II intron RNA is complementary to the nucleotide at +1 in the top strand. If the δ nucleotide is not complementary to the nucleotide at +1, preferably the δ nucleotide is modified to be complementary. The group II intron-encoded protein comprises an RT domain, an X domain, and the conserved and non-conserved regions of a Zn

domain. To insert a cDNA into the cleavage site on the bottom strand of the substrate, the group II intron-encoded protein also comprises a reverse transcriptase domain.

The method of cleaving both strands of a double-stranded DNA sequence having a recognition site comprises the steps of: providing a nucleotide integrase comprising a group II intron RNA having two sequences, EBS1 and EBS2, that are capable of hybridizing with two intron RNA-binding sequences, IBS1 and IBS2, on the top strand of the DNA substrate, and a group II-intron encoded protein that binds to a first sequence element and to a second sequence element in the recognition site of the substrate; and reacting the nucleotide integrase with the double-stranded DNA substrate for a time and at a temperature sufficient to permit the nucleotide integrase to cleave both strands of the DNA substrate and to insert the group II intron RNA into the cleavage site of the top strand. The first sequence element of the recognition site is upstream of the putative cleavage site, the IBS1 sequence and the IBS2 sequence. The first sequence element comprises from about 10 to about 12 pairs of nucleotides. The second sequence element comprises from about 10 to about 12 nucleotides and lies downstream of the cleavage site, i.e., from position +1 to about position +10, +11, or +12.

Nucleotide integrases that may be employed to cleave both strands of a DNA substrate include, but are not limited to an α 2 nucleotide integrase, an α 1 nucleotide integrase, and an ItrA nucleotide integrase. The preferred recognition site for the α 2 nucleotide integrase comprises on the top strand thereof a C at -18, a T at -15, a T at -13, a G at -16 or -19, a T at +1, a T at +4, and a G at +6 relative to the cleavage site. To use the α 2 nucleotide integrase to cleave both strands of the DNA substrate, one first examines the substrate sequence to determine if one strand thereof contains this set of nucleotides. Then, if the EBS2 sequence of the α 2 intron RNA does not have substantial complementarity to the IBS2 sequence of the substrate, i.e., the sequence of 6 nucleotides that lies immediately downstream from the T at -13, and/or if EBS1 sequence of the α 2 intron RNA does not have substantial complementarity to the IBS1 sequence, i.e., the sequence of six nucleotides that lie immediately downstream of the IBS2 sequence and immediately upstream of the T at +1, then the EBS1 sequence and EBS2 sequence of the group II intron RNA are modified to have substantial complementarity, as hereinafter explained. The α 2 nucleotide integrase cleaves both strands of the substrate with greater efficiency if the top strand of the substrate has an A at -21, a G at -19, a C at -18, a G at -16, a T at -15, a T at -13, a T at +1, a T at +4, and a G at +6. The α 2 cleaves both strands of the substrate with even greater efficiency if the top strand has an A at -21, a T at -20, a G at -19, a C at -18, a T at -17, a G at -16, a T at -15, a T at -13, a T at +1, a T at +4, and a G at +6. If the top strand of the substrate additionally has a C at +2, a T at +3, a T at +7, an A at +8, an A at +9, and a T at +10, cleavage will be even greater.

The α 1 integrase is used to cleave both strands of a DNA substrate that has on the top strand thereof a C residue at position -13 relative to the cleavage site a T at +1, a T at +2, a T at +3, a T at +4, an A at +5, a G at +6, a T at +7, and an A at +8 relative to the cleavage site. Preferably, the top strand of the double-stranded substrate has a C at -13, a G at -22, a G at -21, an A at -19, a T at +1, a T at +2, a T at +3, a T at +4, an A at +5, a G at +6, a T at +7, and an A at +8. Cleavage is more efficient if there is a G at -22, a G at -21, an A at -19, an A at +18, a C at -13, a T at +1, an T at +2, an T at +3, a T at +4, a A at +5, a G at +6, a T at +7, an A at +8, a G at +9, and a T at +10 on the top strand of the DNA substrate. If the top strand of the substrate additionally comprises a T at -20, a T at -17, a T at -16, a C at -15, and an A at -14, cleavage will be even greater.

The *ltrA* nucleotide integrase is used to cleave both strands of a double-stranded DNA substrate, when the substrate has on the top strand thereof a G at -21, an A at -20, a C at +1, an A at +2, a T at +3, an A at +4, a T at +5, a C at +6, an A at +7, and a T at +8. The *ltrA* nucleotide integrase cleaves both strands of the substrate more efficiently if the top strand has a G at -21, an A at -20, a T at -19, a G at -17, and G at -15, a C at +1, an A at +2, a T at +3, an A at +4, a T at +5, a C at +6, an A at +7, and a T at +8. If the top strand additionally has a C at -22, a C at -18, a T at -16, an A at -14, an A at -13, a T at +9 and a T at +10, cleavage will be even greater.

Another method uses a nucleotide integrase for cleaving a single-stranded nucleic acid substrate, i.e., a single-stranded DNA or RNA, and for attaching the group II intron RNA molecule into the cleavage site. The method comprises the steps: providing a nucleotide integrase comprising: a group II intron RNA having two hybridizing sequences, EBS1 and EBS2, which are capable of hybridizing with two intron RNA-binding sequences, IBS1 and IBS2, respectively on the substrate, and a group II intron encoded protein having an RT domain, an X domain and the non-conserved portions of the Zn domain; and reacting the substrate with the nucleotide integrase. The EBS1 sequence of the group II intron RNA comprises from about 5 to 7 nucleotides and has at least 80%, preferably 90%, and more preferably full complementarity with the nucleotides at positions -1 to about -5 or about -7. The EBS2 sequence of the group II intron RNA comprises 4 to 7 nucleotides and has at least 80%, preferably 90%, more preferably full complementarity with the nucleotides at positions from about -6 to about -14. Preferably, the nucleotide immediately preceding the first nucleotide of EBS1 is complementary to the nucleotide at +1 in the sense strand.

The present invention also provides a method of determining whether a nucleic acid substrate comprises a particular recognition site. The method comprises the steps of providing a nucleotide integrase capable of cleaving a nucleic acid substrate with a particular recognition site; reacting the nucleotide integrase with the nucleic acid substrate; and assaying for cleavage of the substrate. Cleavage of the substrate indicates that the substrate comprises the particular recognition site. In addition to assaying for fragmentation and alterations in size of the nucleic acid substrate, cleavage may be detected by assaying for incorporation into or attachment of the group II intron RNA to one strand of the nucleic acid substrate.

While a wide range of temperatures are suitable for the methods herein, good results are obtained at a reaction temperature of from about 30°C to about 42°C, preferably from about 30° to about 37°C. A suitable reaction medium contains a monovalent cations such as Na⁺ or K⁺, at a concentration from about 0 to about 300 mM; preferably from about 10 to about 200 mM KCl, and a divalent cation, preferably a magnesium or manganese ion, more preferably a magnesium ion, at a concentration that is less than 100 mM and greater than 1 mM. Preferably the divalent cation is at a concentration of about 5 to about 20mM, more preferably about 10 to about 20 mM. The preferred pH for the medium is from about 6.0-8.5, more preferably about 7.5-8.0.

In the above-described methods it is believed that the single stranded nucleic acid substrates and the top strand of the double-stranded DNA substrate are cut by the excised group II intron RNA. The cleavage that is catalyzed by the excised group II intron RNA is a reverse splicing reaction that results in the insertion, either partially or completely, of the excised group II intron RNA into the cleavage site, i.e. between nucleotides -1 and +1 in the top strand. During partial insertion the group II intron RNA is covalently attached to the +1 nucleotide on the top strand

of the cleavage site. It is believed that the bottom strand or antisense strand of the double-stranded DNA substrate is cut by the group II intron-encoded protein. The bottom strand of the double-stranded DNA substrate is cut at a position from about 9 to about 11 base pairs downstream of the cleavage site in the top strand, i.e., at a site between nucleotide positions +9, +10, and +11.

5 The methods of using a nucleotide integrase as an endonuclease to cleave a substrate DNA are useful analytical tools for determining the presence and location of a particular recognition site in a DNA substrate. Moreover, the simultaneous insertion of a nucleic acid molecule into the DNA substrate, which occurs when either single-stranded DNA or double-stranded DNA is cleaved with a nucleotide integrase, permits tagging of the cleavage site of the DNA substrate with a radiolabeled molecule, a feature which facilitates in identifying DNA substrates that contain a particular recognition site. In addition, the automatic attachment of an RNA molecule onto one strand of a double-stranded DNA substrate permits identification of the cleavage site through hybridization studies that use a probe that is complementary to the attached RNA molecule. An attached RNA molecule that is tagged with a molecule such as biotin also enables the cleaved strand to be affinity purified.

10 The methods of using nucleotide integrases to cleave RNA and DNA substrates having a recognition site are useful for rendering certain genes within the substrates nonfunctional. Such methods are also useful for inserting a nucleic acid into the cleavage site, thus, changing the characteristics of the RNA molecules and the protein molecules encoded by the substrates.

The nucleotide integrase

20 The nucleotide integrase is a ribonucleoprotein ("RNP") particle and comprises a group II intron encoded RNA and a group II intron encoded protein, which protein is bound to the RNA. Preferably, the group II intron RNA is an excised group II intron RNA. "Excised group II intron RNA," as used herein, refers to an RNA that is either an *in vitro* or *in vivo* transcript of the DNA of the group II intron and that lacks flanking exon sequences. The excised group II intron RNA is obtained from wild type organisms, or mutated organisms, by *in vivo* transcription and splicing, or by *in vitro* transcription and splicing from the transcript of a modified or unmodified group II intron. "Group II intron encoded protein" as used herein, is a protein encoded by a group II intron open reading frame.

25 Group II introns are a specific type of intron which is present in the DNA of bacteria and in the DNA of organelles, particularly the mitochondria of fungi, yeast and plants and the chloroplast of plants. The group II intron RNA molecules, that is, the RNA molecules which are encoded by the group II introns, share a similar secondary and tertiary structure. Figure 2 depicts the secondary structure of the a11 and a12 intron RNA and part of the nucleotide sequence of the wild-type a11 and a12 intron RNA. The group II intron RNA molecules typically have six domains. Domain IV of the group II intron RNA contains the nucleotide sequence which encodes the "group II intron encoded protein."

30 Nucleotide integrases include, for example, excised group II intron RNA molecules having a sequence which is identical to a group II intron RNA that is found in nature, i.e. a wild-type group II intron RNA, and excised group II intron RNA's which have a sequence different from a group II intron RNA that is found in nature, i.e. a modified, excised group II intron RNA molecule. Modified excised group II intron RNA molecules, include, for

example, group II intron RNA molecules that have nucleotide base changes or additional nucleotides in the internal loop regions of the group II intron RNA, preferably the internal loop region of domain IV and group II intron RNA molecules that have nucleotide base changes in the hybridizing regions of domain I. Nucleotide integrases in which the group II intron RNA has nucleotide base changes in the hybridizing region, as compared to the wild type, typically have altered specificity for the substrate DNA of the nucleotide integrase.

The group II intron-encoded protein of the nucleotide integrase comprises an X domain and a Zn domain. The X domain of the protein has a maturase activity. The Zn domain of the protein has Zn²⁺ finger-like motifs. Preferably, the group II intron-encoded protein further comprises a reverse transcriptase domain. As used herein, a group II intron-encoded protein includes modified group II intron-encoded proteins that have additional amino acids at the N terminus, or C terminus, or alterations in the internal regions of the protein as well as wild-type group II intron-encoded proteins. It is believed that the group II intron-encoded protein is bound to 3' region of the group II intron RNA.

The nucleotide integrase are provided in the form of RNP particles isolated from wild-type, mutant, or genetically-engineered organisms. The nucleotide integrase are also provided in the form of reconstituted RNP particles isolated from a reconstituted RNP particle preparation. The nucleotide integrase also comprises reconstituted RNP particles that are formed by combining an exogenous synthetic, excised group II intron RNA with either a group II intron-encoded protein or an RNA-protein complex preparation. The exogenous RNA includes both unmodified and modified group II intron RNA molecules. Preferably, the exogenous RNA is an *in vitro* transcript or a derivative of an *in vitro* transcript of an unmodified or modified group II intron. For example, the exogenous RNA may be derived by splicing from an *in vitro* transcript. The RNA-protein complex preparation contains group II intron-encoded protein molecules complexed to RNA molecules that are not an excised group II RNA molecule having a sequence which encodes this protein. The group II intron-encoded protein of the RNA-protein complex is associated with either a ribosomal RNA molecule, an mRNA molecule, or an excised group II intron RNA that does not encode the group II-intron encoded protein.

The nucleotide integrase may be used as a purified RNP particle or a purified reconstituted particle. Alternatively, the nucleotide integrase may be used in a partially-purified preparation which contains the RNP particles and reconstituted particles that have nucleotide integrase activity as well as other RNP particles, such as for example ribosomes. This partially-purified preparation is free of organelles.

Preparation of the Nucleotide Integrase

The nucleotide integrase is isolated from wild type or mutant yeast mitochondria, fungal mitochondria, plant mitochondria, chloroplasts, the proteobacterium *Azotobacter vinelandii*, the cyanobacterium *Calothrix*, and *Escherichia coli lactococcus lactis*. The procedure for isolating the RNP particle preparation involves mechanically and/or enzymatically disrupting the cell membranes and/or cell walls of the organisms. In the case of fungi and plants, the purification also involves separating the specific organelles, such as mitochondria or chloroplasts, from the other cellular components by differential centrifugation and/or flotation gradients and then lysing the organelles with a nonionic detergent, such as Nonidet P-40. The organelle and bacterium lysates are then centrifuged

through a sucrose cushion to obtain the ribonucleoprotein (RNP) particle preparation. The RNP particles may be further purified by separation on a sucrose gradient, or a gel filtration column, or by other types of chromatography.

The nucleotide integrase is also isolated from reconstituted RNP particle preparations that are prepared by combining an RNA-protein complex preparation with an exogenous, excised group II intron RNA. The RNA-protein complex preparation is preferably isolated from a yeast, fungi, or bacterium using the protocol for RNP particles described above. The RNA-protein complex preparation comprises group II intron-encoded protein molecules complexed with RNA molecules that are not an excised group II intron RNA having a sequence that encodes the group II intron-encoded protein. The group II intron-encoded protein of the RNA-protein complex preparation is associated with either a ribosomal RNA molecule, an mRNA molecule, or an excised group II intron RNA that does not encode the group II-intron-encoded protein.

The exogenous RNA preferably is a synthetic molecule made by in vitro transcription or by in vitro transcription and self-splicing of the group II intron. The exogenous RNA may also be made by isolation of the group II intron RNA from cells or organelles in which it is naturally present or from cells in which an altered intron has been inserted and expressed. The exogenous RNA is then added to a preparation containing the RNA-protein complex. Preferably, the exogenous group II intron RNA is first denatured. The exogenous RNA is added to the RNA-protein complex on ice.

In another embodiment, the nucleotide integrase is made by introducing an isolated DNA molecule which comprises a group II intron DNA sequence into a host cell. Suitable DNA molecules include, for example, viral vectors, plasmids, and linear DNA molecules. Following introduction of the DNA molecule into the host cell, the group II intron DNA sequence is expressed in the host cell such that excised RNA molecules encoded by the introduced group II intron DNA sequence and protein molecules encoded by introduced group II intron DNA sequence are formed in the cell. The excised group II intron RNA and group II intron-encoded protein are combined within the host cell to produce the nucleotide integrase.

Preferably the introduced DNA molecule also comprises a promoter, more preferably an inducible promoter, operably linked to the group II intron DNA sequence. Preferably, the DNA molecule further comprises a sequence which encodes a tag to facilitate isolation of the nucleotide integrase such as, for example, an affinity tag and/or an epitope tag. Preferably, the tag sequences are at the 5' or 3' end of the open reading frame sequence. Suitable tag sequences include, for example, sequences which encode a series of histidine residues, the Herpes simplex glycoprotein D, i.e., the HSV antigen, or glutathione S-transferase. Typically, the DNA molecule also comprises nucleotide sequences that encode a replication origin and a selectable marker. Optionally, the DNA molecule comprises sequences that encode molecules that modulate expression, such as for example T7 lysozyme.

The DNA molecule comprising the group II intron sequence is introduced into the host cell by conventional methods, such as, by cloning the DNA molecule into a vector and by introducing the vector into the host cell by conventional methods, such as electroporation or by CaCl_2 -mediated transformation procedures. The method used to introduce the DNA molecule is related to the particular host cell used. Suitable host cells are those which are capable of expressing the group II intron DNA sequence. Suitable host cells include, for example, heterologous or homologous bacterial cells, yeast cells, mammalian cells, and plant cells. In those instances where the host cell genome

and the group II intron DNA sequence use different genetic codes, it is preferred that the group II intron DNA sequence be modified to comprise codons that correspond to the genetic code of the host cell. The group II intron DNA sequence, typically, is constructed de novo from synthetic oligonucleotides or modified by *in vitro* site-directed mutagenesis to prepare a group II intron DNA sequence with different codons. Alternatively, to resolve the differences in the genetic code of the intron and the host cell, DNA sequences that encode the tRNA molecules which correspond to the genetic code of the group II intron are introduced into the host cell. Optionally, DNA molecules which comprise sequences that encode factors that assist in RNA or protein folding, or that inhibit RNA or protein degradation are also introduced into the cell.

The DNA sequences of the introduced DNA molecules are then expressed in the host cell to provide a transformed host cell. As used herein the term "transformed cell" means a host cell that has been genetically engineered to contain additional DNA, and is not limited to cells which are cancerous. Then the RNP particles having nucleotide integrase activity are isolated from the transformed host cells.

Preferably, the nucleotide integrase is isolated by lysing the transformed cells, such as by mechanically and/or enzymatically disrupting the cell membranes of the transformed cell. Then the cell lysate is fractionated into an insoluble fraction and soluble fraction. Preferably, an RNP particle preparation is isolated from the soluble fraction. RNP particle preparations include the RNP particles having nucleotide integrase activity as well as ribosomes, mRNA and tRNA molecules and other RNPs. Suitable methods for isolating RNP particle preparations include, for example, centrifugation of the soluble fraction through a sucrose cushion. The RNP particles, preferably, are further purified from the RNP particle preparation or from the soluble fraction by, for example, separation on a sucrose gradient, or a gel filtration column, or by other types of chromatography. For example, in those instances where the protein component of the desired RNP particle has been engineered to include a tag such as a series of histidine residues, the RNP particle may be further purified from the RNP particle preparation by affinity chromatography on a matrix which recognizes and binds to the tag. For example, NiNTA Superflow from Qiagen, Chatsworth CA, is suitable for isolating RNP particles in which the group II intron-encoded protein has a His₆ tag.

The following methods for preparing nucleotide integrases are included for purposes of illustration and are not intended to limit the scope of the invention.

FORMULATIONS

The RNP particle preparations of the following formulations 1-10, and the RNA-protein complex of the formulation 12 were isolated from the mitochondria of the wild-type *Saccharomyces cerevisiae* yeast strain ID41-6/161 *MATa adel lys1*, hereinafter designated "161", and derivatives thereof. The mitochondria of the wild-type yeast strain 161 contains a *COX1* gene that includes the group II intron aI1 and the group II intron aI2.

The *COX1* gene in the mutant yeast strains either lacks one of the group II introns or has a mutation in one of the group II introns. The excised group II intron RNA molecules and the group II intron encoded proteins are derived from the group II introns aI1 and aI2 that are present in the wild-type and mutant yeast strains.

The intron composition of the *COX1* gene in the different yeast strains is denoted by a convention in which a superscript "+" indicates the presence of the a11 intron or the a12 intron, a superscript "0" indicates the absence of the a11 or a12 intron, and other superscripts refer to specific alleles or mutations in the a12 intron.

Formulation 1

5 An RNP particle preparation was isolated from the mitochondria of the *Saccharomyces cerevisiae* wild-type yeast strain 161. The intron composition of the *COX1* gene of the wild-type strain is 1²⁺. The RNP particle preparation contains an RNP particle that is derived from the a11 intron and includes an excised a11 RNA bound to a protein encoded by a11. The preparation also contains an RNP particle that is derived from the a12 intron and that comprises a excised a12 RNA molecule and an associated a12-encoded protein.

10 To prepare the RNP particle preparation, the yeast were inoculated into a 1 liter liquid culture medium containing 2% raffinose, 2% BactoPeptone from Difco and 1% yeast extract from Difco to an O.D.₅₉₅ of 1.6-1.7. The cell walls were digested with 40 mg of the yeast lytic enzyme from ICN, and the cells broken by mechanical disruption with glass beads. The nuclei and cell debris were pelleted from the lysate by centrifugation for 5 minutes in a Beckman GSA rotor at 5,000 rpm. The supernatant was removed and centrifuged in a Beckman GSA rotor at 13,000 rpm for 15 minutes to obtain a mitochondrial pellet. The mitochondria were layered on a flotation gradient consisting of a 44% sucrose solution layer, a 53% sucrose solution layer, and a 65% sucrose solution layer and centrifuged in a Beckman SW28 rotor at 27,000 rpm for 2 hours and 10 minutes. The mitochondria were collected from the 53%/44% interface and suspended in buffer containing 0.5M KCl, 50 mM CaCl₂, 25 mM Tris-HCl, pH 7.5, 5 mM DTT and lysed by the addition of Nonidet P-40 to a final concentration of 1%. The mitochondrial lysate was then centrifuged in a Beckman 50Ti rotor at 50,000 rpm for 17 hours through a 1.85 M sucrose cushion in a buffer containing 0.5M KCl, 25 mM CaCl₂, 25 mM Tris-HCl, pH 7.5, 5 mM DTT, to obtain a pellet of RNP particles that were largely free of mitochondrial proteins. The isolated RNP particles were resuspended in 10 mM Tris-HCl, pH 8.0 and 1 mM DTT and stored at -70°C. The preparation may be repeatedly thawed and frozen before use.

Formulation 1a Purified RNP particle

25 2.5 O.D.₂₆₀ of the RNP particles from formulation 1 in a volume of 150 µl were layered onto a 12 ml 5-20% linear sucrose gradient in a buffer consisting of 100 mM KCl, 2 mM MgCl₂, 50 mM Tris-HCl, pH 7.5, and 5 mM DTT. The gradient was centrifuged in an SW41 rotor at 4°C at 40,000 rpm for five hours. The gradient was fractionated into 35 fractions of approximately 0.325 ml. Fractions 12-20 contain the purified RNP particles which are substantially free of ribosomal RNA. The location of the RNP particles in the gradient fractions was independently verified by Northern hybridization with a12 antisense RNA. The location of the small and large subunits of ribosomal RNA in the gradient fractions was independently verified by ethidium bromide staining of the fractions on a 1% agarose gel. Approximately 85% of the ribosomal RNA is found in a fraction that does not contain the RNP particles which comprise the nucleotide integrase.

Formulation 2 RNP particle preparation from mutant yeast strain 1²⁺

35 The RNP particles comprise an excised a12 RNA and an a12-encoded protein. Yeast strain 1²⁺ was obtained from Dr. Philip S. Perlman at the University of Texas Southwestern Medical Center and was prepared as

described in Moran et al., 1995, Mobile Group II Introns of Yeast Mitochondrial DNA Are Novel Site-Specific Retroelements, Mol. Cell Biol. 15, 2828-38, which is incorporated herein by reference. The 1[°]2[°] mutant strain was constructed as follows: (i) the aI2 intron from strain 161 was cloned as a *Clal*-to-*Bam*HI fragment into pBluescript KS⁺ obtained from Stratagene to yield pJVM4; (ii) pJVM4 was cleaved with *Clal* and *Nde*I to remove the 5' end of the insert; and (iii) an *Msp*I-to-*Nde*I fragment that contains exons 1 and 2 of the mitochondriae *COX1* gene plus the 5' end of aI2 from yeast strain C10361Δone was inserted to yield plasmid pJVM164. Yeast strain C10361Δone, in which aI1 is excised from the mitochondrial DNA, was prepared as described in Kennell et al., 1993, Reverse transcriptase activity associated with maturase-encoding group II introns in yeast mitochondria. Cell 73, 133-146, which is incorporated herein by reference. pJVM164 was transformed into a [*rho*⁰] strain, and the 1[°]2[°] allele was placed into an intact mitochondrial DNA by recombination. This last step is accomplished by mating to a nonreverting *COX1* mutant derived from mutant C1036 (strain 5B), whose construction is described in Kennel et al., 1993, and selecting for recombinant progeny that are capable of respiring and growing on glycerol-containing medium (GLY⁺) and that contain the transformed *COX1* allele in place of the 5B allele.

The reactions and manipulations directed at cloning DNA, such as ligations, restriction enzyme digestions, bacterial transformation, DNA sequencing etc. were carried out according to standard techniques, such as those described by Sambrook et al., Molecular cloning: a laboratory manual, 2nd ed., Cold Spring Harbor Laboratory Press. Cold Spring Harbor, N.Y. Yeast mitochondrial transformations were also carried out according to standard techniques such as those described in Belcher et al., 1994, Biolistic transformation of mitochondria in *Saccharomyces cerevisiae*, 101-115. In N.-S. Yang and P. Christou (ed.) Particle Bombardment Technology for Gene Transfer. Oxford University Press, New York. The RNP particle preparation was made from the mitochondria of mutant yeast strain 1[°]2[°], as in formulation 1.

Formulation 3 RNP particle preparation from mutant yeast strain 1[°]2[°]

Yeast strain 1[°]2[°] is a derivative of the wild-type yeast strain 161. The yeast strain 1[°]2[°] was obtained from Dr. Philip S. Perlman and was prepared as described in Kennell et al., 1993. Cell 73, 133-146. Yeast strain 1[°]2[°] contains a segment of the *COX1* gene of *S. diastaticus*, which lacks aI2, inserted into wild-type 161 mtDNA via mitochondrial transformation. The construction started with plasmid pSH2, which contains aI1 from wild-type 161 and some flanking sequences cloned as a *Hpa*II/*Eco*RI fragment in pBS(+) (Stratagene, La Jolla, CA). That plasmid was cleaved near the 3' end of aI1 with *Clal* and in the downstream polylinker with *Bam*HI, and the gap was filled with a *Clal*/*Bam*HI fragment from *S. diastaticus* mitochondrial DNA (NRRL Y-2416) that contains the 3' end of aI1, E2, E3 and most of aI3, thus creating a 1[°]2[°] form of the *COX1* gene. The plasmid containing the hybrid *COX1*-1[°]2[°] segment was transformed into a *rho*⁰ derivative of strain MCC109 (*MATα ade2-101 ura3-52 kar1-1*) by biolistic transformation. The resulting artificial petite was crossed to strain n161/m161-5B, and gly⁺ recombinants containing the *COX1* 1[°]2[°] allele in the n161 background were isolated. The hybrid aI1 allele, which is spliced normally, differs from that of wild-type 161 by one nucleotide change, C to T, at position 2401, changing Thr₇₄₄ to Leu in the intron open reading frame. The RNP particle preparation was made from the mitochondria of mutant yeast strain 1[°]2[°] as in formulation 1. The RNP particles comprise an excised aI1 RNA molecule and an aI1 encoded protein.

Formulation 4 RNP particle preparation from mutant yeast strain 1[°]2^{YASH}

Yeast strain 1°2^{YAHH} was obtained from Dr. Philip S. Perlman and was made as described in Moran et al., 1995, Mol. Cell Biol. 15, 2838-38., using a mutagenized pJVM164 plasmid. The allele was made by oligonucleotide-directed mutagenesis of pJVM164 which contains a 4.4 kb MspI/BamHI fragment extending from 217 nucleotides upstream of exon 1 through intron a13 of the COX1 allele. The mutagenesis changes the a12 nucleotides 1473 to 1478 from GAT GAT to CAT CAT (D-491D-492 to HH). The RNP particles comprise a mutated, excised a12 RNA and an a12-encoded protein that has the mutation YADDYAAH in the reverse transcriptase domain of the protein. The RNP particle preparation was made from the mitochondria of mutant yeast strain 1°2^{YAHH} as in formulation 1.

Formulation 5 RNP particles from the mutant yeast strain 1°2^{P714T}

The mutant yeast strain 1°2^{P714T} was obtained from Dr. Philip S. Perlman and was constructed according to the procedure described in Kennell et al., 1993, Cell 73, 133-146, where it is named n161/m161-C1036Δ1. The RNP particles comprise a mutated, excised a12 intron RNA molecule and an a12-encoded protein that carries the missense mutation P₇₁₄T in the Zn domain. The RNP particle preparation was made from mitochondria of mutant yeast strain 1°2^{P714T} as in formulation 1.

Formulation 6 RNP particle from mutant yeast strain 1°2^{HHVR}

The mutant yeast strain 1°2^{HHVR} was obtained from Dr. Philip S. Perlman and was made by using the nucleotide described in Moran et al., 1995, Mol. Cell Biol. 15, 2828-38, which is incorporated herein by reference, using a mutagenized pJVM164 plasmid. The allele was constructed by site-directed mutagenesis of pJVM164. The a12 intron has the following changes: positions 2208-2219 from CATCACGTAAGA SEQ. ID. NO. 9 to GCAGCTGCAGCT, (H₇₃₆H₇₃₇V₇₃₈R₇₃₉ to AAAA) and A₂₂₂₇ A to T (N₇₄₂I). This nucleotide integrase preparation comprises a mutated, excised a12 intron RNA and an a12-encoded protein that has a missense mutation in the HHVR motif. The RNP particle preparation was made from mitochondria of mutant yeast strain 1°2^{HHVR}.

Formulation 7 RNP particle from mutant yeast strain 1°2^{ΔConZn}

The mutant yeast strain 1°2^{ΔConZn} was obtained from Dr. Philip S. Perlman and was made as described in Moran et al., 1995, Mol. Cell Biol. 15, 2828-38, using a mutagenized pJVM164 plasmid. The allele was constructed by oligonucleotide-directed mutagenesis of pJVM164. The a12 intron has the following changes: positions 2157-2165 changed from TTATTTAGT to TAATAATAA (L₇₁₉F₇₂₀S₇₂₁ to OchOchOch). RNP particles comprise a mutated, excised a12 intron RNA and an a12-encoded protein that lacks the most conserved motifs in the Zn domain. The RNP particle preparation was made from mitochondria of mutant yeast strain 1°2^{ΔConZn}.

Formulation 8 RNP particle from mutant yeast strain 1°2^{C-C1}

The mutant yeast strain 1°2^{C-C1} was obtained from Dr. Phillip S. Perlman and was made by using a nucleotide described in Moran et al., 1995 Mol. Cell Biol. 15, 2828-38, using a mutagenized pJVM164 plasmid. The allele was constructed by site-directed mutagenesis of pJVM164. The a12 intron has the following changes: positions 2172-2173 changed from TG to GC (C₇₂₄A) and 2180-2182 changed from TTG to AGC (I₇₂₆C₇₂₇ to MA). The RNP particles comprise a mutated, excised a12 intron RNA and an a12-encoded protein that has three amino acid residues changed in the first Zn²⁺-finger-like motif. The RNP particle preparation was made from mitochondria of mutant yeast strain 1°2^{C-C1}.

Formulation 9 RNP particles from mutant yeast strain 1^o2^{C-C2}

The mutant yeast strain 1^o2^{C-C2} was obtained from Dr. Philip S. Perlman and was made as described in Moran et al., 1995 Mol. Cell Biol. 15,2828-38, using a mutagenized pJVM164 plasmid. The allele was constructed by site-directed mutagenesis of pJVM164. The aI2 intron has the following changes: position 2304-2305 changed from TG to GC (C₇₆₈A) and 2313-2314 changed from TG to GC (C₇₇₁A). The RNP particles comprise a mutated excised aI2 intron RNA and an aI2-encoded protein that has two amino acids changed in the second Zn⁺² finger-like motif. The RNP particle preparation was made from mitochondria of mutant yeast strain 1^o2^{C-C2}.

Formulation 10 RNP particles from mutant yeast strain 1^o2^{H6}

The mutant yeast strain, obtained from Dr. Philip S. Perlman, was made by transferring the mutagenized plasmid pJVM164 into the mitochondria of yeast strain GRF18 as described in Moran et al., 1995 Ref. The allele was constructed by site-directed mutagenesis of pJVM164 and has the sequence CATCATCATCATCAT, SEQ. ID. NO. 10, inserted between nucleotides 2357 and 2358 of the aI2 intron. The RNP particle preparation was made from mitochondria of mutant yeast strain 1^o2^{H6} according to the protocol described above for formulation 1. The RNP particles comprise a mutated, excised aI2 intron RNA and an aI2-encoded protein that has six histidines added to the C terminus of the aI2-encoded protein.

Formulation 11 RNP particles from Neurospora intermedia.

Mitochondria from the Varkud strain of *Neurospora intermedia*, which is available from the Fungal Genetics Stock Center, were prepared as described in Lambowitz A.M. 1979, Preparation and analysis of mitochondrial ribosomes. Meth. Enzymol. 59, 421-433. The conidia were disrupted with glass beads and the mitochondria and RNP particles isolated as described in formulation 1. The RNP particles comprise an excised *col* intron RNA and the protein encoded by the *col* intron.

Formulation 12 Reconstituted RNP particle preparation

A reconstituted RNP particle preparation was made by incubating an exogenous, excised, in vitro RNA transcript of the aI2 intron with an RNA-protein complex preparation isolated from the mutant yeast strain 1^o2^{AD5}, in which the aI2 intron RNA lacks a domain V and is therefore splicing defective. The mutant allele 1^o2^{AD5} was obtained from Dr. Philip S. Perlman and was constructed using the same procedure that was used to make yeast strain 1^o2^{AD5} that was described in Moran et al. 1995, except that the final mating was with yeast strain 1^o2⁺. The RNA-protein complex preparation was isolated from 1^o2^{AD5} using the protocol described above in formulation 1 for RNP particle preparations. The RNA-protein complex preparation isolated from the mitochondria of 1^o2^{AD5} does not contain excised aI2 RNA but does contain aI2-encoded protein that is associated with other RNA molecules in the preparation.

The exogenous RNA was made by in vitro transcription of the plasmid pJVM4 which includes a fragment of the yeast mitochondrial *COX1* gene from the *Clal* site of the group II intron 1 (aI1) to the *BamHI* site of aI3 that has been inserted into the pBLUESCRIPT KS(+) plasmid. Plasmid pJVM4 contains the following *COX1* sequences: Exon 2, aI2, Exon 3 and parts of aI1 and aI3 sequence. The sequences are operably linked to a T3 RNA polymerase promoter. The Exon 2 and Exon 3 sequence are required for self-splicing of the aI2 intron RNA from the

RNA transcript. pJVM4 was linearized with *BstEII*, which cuts at the 3' end of Exon 3 then 5 µg of the plasmid was incubated in 0.300 ml of 40 mM Tris-HCl at pH 8.0, 25 mM NaCl, 8 mM MgCl₂, 2 mM spermidine, 5 mM DTT 500 mM rNTPs, 600 U of RNasin from US Biochemical and 300-750 U of T3 RNA polymerase from BRL at 37°C for 2 hours to make the RNA transcripts. Following the incubation, the RNA transcripts were phenol-CIA extracted, purified on G-50 column, phenol-CIA extracted and precipitated with ethanol. The RNA transcripts were then incubated in 40 mM Tris-HCl at pH 7.5, 100 mM MgCl₂, 2 M NH₄Cl at 40-45°C for 1 hour to allow self-splicing of the aI2 intron RNA molecules from the RNA transcripts and to obtain the splicing products. The splicing products, which include the excised aI2 RNA transcript, the ligated transcript which lacks the aI2 intron RNA, and the unspliced transcript, were desalted by passing through a G-50 column, then phenol-CIA extracted and ethanol precipitated to provide the exogenous RNA. The exogenous RNA was then resuspended to a final concentration of 1.0 µg/µl in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA.

To prepare the reconstituted RNP particle preparation, 1 µl of the exogenous RNA was added to 2 µl of the 1°^{240S} RNA-protein complex preparation (0.025 O.D.₂₆₀ units) on ice for 0-10 minutes. The preparation was used immediately.

15 Formulation 13 Reconstituted RNP Particle Preparation containing a Nucleotide Integrase Comprising a Group II Intron RNA Having Modified EBS Sequences

Plasmid pJVM4 derivatives were used to prepare exogenous aI2 intron RNA molecules in which the EBS1 and EBS2 sequences are different from the EBS sequences in the wild-type aI2 intron. pJVM4 contains the aI2 intron sequence and flanking exon sequences from wild-type yeast 161 cloned downstream of a phage T3 promoter in pBluescript II KS(+). Plasmids containing modified introns were derived from pJVM4 by PCR mutagenesis with appropriate primers. In all cases, the modified region was sequenced to verify the correct mutation and the absence of adventitious mutations.

Plasmids pJVM4-aI1EBS1, pJVM4-aI1EBS2 and pJVM4-aI1EBS1/EBS2 contain aI2 RNA derivatives in which the EBS1 and/or EBS2 sequences were replaced with those of aI1. In each case, portions of the 5' and 3' exons were also changed to aI1 sequences to permit in vitro splicing. pJVM4-aI1EBS1 has EBS1 positions 2985-2990 changed from 5'AGAAGA to 5'CGTTGA; pJVM4-aI1EBS2 has EBS2 positions 2935-2940 changed from 5' TCATTA to 5' ACAATT; and pJVM4-aI1EBS1EBS2 has EBS1 and EBS2 positions 2935-2940 and 2985-2990 changed from 5' TCATTA to 5' ACAATT and 5' AGAAGA to 5' CGTTGA, respectively. For pJVM4-aI1EBS1 and pJVM4-aI1EBS1/EBS2, the 5' portion of the pJVM4 insert consisting of aI1 and E2 sequence was replaced with the last 24 bp of E1. For pJVM4-aI1EBS2, positions -24 to -7 (GTCATGCTGTATTAATGA) SEQ. ID. NO. 11 were replaced with (ATGGTAATTCACAATTAT), SEQ. ID. NO. 12 leaving the aI2 EBS1 sequence unchanged. For all three constructs, the 3' portion of the insert was replaced by the first 15 bp of E2 instead of E3 and aI3.

pJVM4-EBS2-8G, pJVM4-EBS2-9T-10A, pJVM4-EBS2-11A, pJVM4-EBS2-12T, and pJVM4-EBS2-13T(1) are derivatives of pJVM4 in which the indicated changes were introduced at different positions in EBS2. pJVM4-EBS2-13T(2) is identical to pJVM4-EBS2-13T(1) except that it contains a second mutation, T to A, at intron position 2932.

pJVM4- δ -C, pJVM4- δ -G, and pJVM9- δ -T are derivatives of pJVM4 in which the δ nucleotide (position 2984) was changed to C, G, or T, respectively, with the compensatory nucleotide substituted at the δ' position of exon 3 for in vitro splicing.

Exogenous aI2 intron transcripts having a modified EBS1 sequence and/or a modified EBS2 sequence were synthesized using phage T3 polymerase and the modified plasmids as templates. The synthetic transcripts contained regions of the modified aI2 intron RNA and regions of the flanking exon 2 and exon 3 of the yeast mitochondrial COX1 protein. The synthetic transcripts were self-spliced and the spliced products desalted through a G-50 column, phenol-CIA extracted, ethanol precipitated, and dissolved in TE (pH8.0) at a final concentration of 1.0 μ g/ μ l (0.52 μ M).

The resulting modified, excised aI2 RNA molecules were individually mixed with RNA-protein complex preparations isolated from 1^o2^{AD5} using the protocol described above in formulation 1 for RNP particle preparations. This yeast mutant has a deletion in domain V of the aI2 intron and is unable to splice aI2 RNA. This mutant overproduces aI2 protein from the unspliced precursor mRNA. Thus, the RNA-protein complex preparation contains larger amounts of the aI2 protein.

For reconstitution, 1 μ l of the spliced, synthetic aI2 transcripts was mixed with 2 μ l (0.025OD₂₆₀ units) of the RNA-protein complex preparation and incubated on ice for 0-10 minutes.

Formulation 14

An RNP particle preparation containing an RNP particle in which the loop region of domain IV of the group II intron RNA is modified, that is the loop region nucleotide sequence of domain IV differs from the nucleotide sequence of the aI2 RNA of formulations 1-10 is prepared by two methods. First oligonucleotide-directed mutagenesis of the aI2 intron DNA is performed by standard, well-known methods to change the nucleotide sequences which encode for the loop region of domain IV of the aI2 intron RNA. The mutagenized aI2 intron DNA is then inserted into a vector, such as a plasmid, where it is operably linked to an RNA polymerase promoter, such as a promoter for T7 RNA polymerase or SP6 RNA polymerase or T3 RNA polymerase and an in vitro transcript of the modified group II intron RNA is made as described above in formulation 12. The exogenous RNA is then combined with an RNA-protein complex that has been isolated as described for formulation 12 to produce a modified reconstituted RNP particle preparation.

Alternatively, an RNP particle preparation in which the sequences within the loop region of the group II intron RNA are modified is prepared by site-directed mutagenesis of an organism, such as a yeast, as described in formulations 4-10, and by isolation of the RNP particle preparation from the organism as described in formulation 1.

Formulation 15 RNP Particle Preparation from a Genetically-Engineered Cell

A nucleotide integrase comprising an excised RNA which is encoded by the LI.ltrB intron of a lactococcal conjugative element pRS01 of *Lactococcus lactis* and the protein encoded by the ORFLtrA of the LI.ltrB intron were prepared by transforming cells of the BLR(DE3) strain of the bacterium *Escherichia coli*, which has the *recA* genotype, with the plasmid pETLtrA19. Plasmid pETLtrA19 comprises the DNA sequence for the group II

intron LI.ltrB from *Lactococcus lactis*, positioned between portions of the flanking exons *ltrBE1* and *ltrBE2*. pETLtrA19 also comprises the DNA sequence for the T7 RNA polymerase promoter and the T7 transcription terminator. The sequences are oriented in the plasmid in such a manner that the ORF sequence, SEQ. ID. NO. 6, within the LI.ltrB intron is under the control of the T7 RNA polymerase promoter. The ORF of the LI.ltrB intron encodes the protein *ltrA*. The sequence of the LI.ltrB intron and the flanking exon sequences present in pETLtrA19 are shown SEQ.ID. NO. 5. The amino acid sequence of the *ltrA* protein is shown in SEQ. ID. NO.7. Domain IV is encoded by nucleotide 705 to 2572.

pETLtrA19 was prepared first by digesting pLE12, which was obtained from Dr. Gary Dunny from the University of Minnesota, with *HindIII* and isolating the restriction fragments on a 1% agarose gel. A 2.8 kb *HindIII* fragment which contains the LI.ltrB intron together with portions of the flanking exons *ltrBE1* and *ltrBE2* was recovered from the agarose gel and the single-stranded overhangs were filled in with the Klenow fragment of DNA polymerase I obtained from Gibco BRL, Gaithersburg, MD. The resulting fragment was ligated into plasmid pET-11a that had been digested with *XbaI* and treated with Klenow fragment. pET-11a was obtained from Novagen, Madison, WI.

pETLtrA19 was introduced into the *E. coli* cells using the conventional CaCl_2 -mediated transformation procedure of Sambrook et al. as described in "Molecular Cloning A Laboratory Manual", pages 1-82, 1989. Single transformed colonies were selected on plates containing Luria-Bertani (LB) medium supplemented with ampicillin to select the plasmid and with tetracycline to select the BLR strain. One or more colonies were inoculated into 2 ml of LB medium supplemented with ampicillin and grown overnight at 37°C with shaking. 1 ml of this culture was inoculated into 100 ml LB medium supplemented with ampicillin and grown at 37° C with shaking at 200 rpm until OD_{595} of the culture reached 0.4. Then isopropyl-beta-D-thiogalactoside was added to the culture to a final concentration of 1 mM and incubation was continued for 3 hours. Then the entire culture was harvested by centrifugation at 2,200 x g, 4°C, for 5 minutes. The bacterial pellet was washed with 150 mM NaCl and finally resuspended in 1/20 volume of the original culture in 50 mM Tris, pH 7.5, 1 mM EDTA, 1 mM DTT, and 10% (v/v) glycerol (Buffer A). Bacteria were frozen at -70°C.

To produce a lysate the bacteria were thawed and frozen at -70°C three times. Then 4 volumes of 500 mM KCl, 50 mM CaCl_2 , 25 mM Tris, pH 7.5, and 5 mM DTT (HKCTD) were added to the lysate and the mixture was sonicated until no longer viscous, i.e. for 5 seconds or longer. The lysate was fractionated into a soluble fraction and insoluble fraction by centrifugation at 14,000 x g, 4°C, for 15 minutes. Then 5 ml of the resulting supernatant, i.e., the soluble fraction, were loaded onto a sucrose cushion of 1.85 M sucrose in HKCTD and centrifuged for 17 hours at 4°C, 50,000 rpm in a Ti 50 rotor from Beckman. The pellet which contains the RNP particles was washed with 1 ml water and then dissolved in 25 μl 10 mM Tris, pH 8.0, 1 mM DTT on ice. Insoluble material was removed by centrifugation at 15,000 x g, 4°C, for 5 minutes. The yield of RNP particles prepared according to this method comprise the excised LI.ltrB intron RNA and the *ltrA* protein.

Preparation of Substrate DNA

Labeled DNA substrates having sequences from the E2/E3 junction of the yeast mitochondrial COX1 gene, the E1/E2 junction of the yeast mitochondrial COX1 gene, and the E1/E2 junction of the putative *Lactococcus lactis* relaxase gene (*ltrB*) were synthesized from recombinant plasmids or synthetic oligonucleotide templates by PCR or primer extension. The sequence of the substrate containing the E2/E3 junction of the yeast mitochondrial COX 1 gene is depicted in Figure 3 as the wt sequence. Figure 3 also identifies the locations of the mutations in this sequence. The sequence of the substrate containing the E1/E2 junction of the yeast mitochondrial COX 1 gene is depicted in Figure 6, which also identifies the locations of the mutations in this sequence. The sequence of the substrate containing the E1/E2 junction of the putative *Lactococcus lactis* relaxase gene (*ltrB*) is depicted in Figure 8, which also identifies the locations of the mutations in this sequence. DNA substrates that were labeled on the 5' end of the antisense strand were also generated from plasmids by PCR with 200 ng of the 5' end-labeled primer and unlabeled primer, both of which are complementary to a sequence in the polylinker. Single-stranded DNA substrates were synthesized by end-labeling nucleotides. Short segments of double-stranded DNA substrates were also prepared

The following examples of methods employing nucleotide integrases comprising an excised *al2* intron RNA bound to an *al2* protein, an excised *al1* intron RNA bound to an *al1* protein, or an excised *ltrA* intron RNA bound to an *ltrA* protein to cleave DNA substrates are for illustration only and are not intended to limit the scope of the invention.

Example 1 Cleaving a Double-Stranded DNA Substrate with a Nucleotide Integrase Comprising a wild-type *al2* Intron RNA and a wild-type *al2*-Encoded Protein

0.025 O.D.₂₆₀ units of the RNP particles of formulation 1 were reacted with a DNA substrate consisting of yeast mitochondrial *COX1* exons 2 and 3 (E2E3) and comprising the WT sequence shown in Figure 3. The reaction was conducted at 37°C in a buffer containing 100 mM KCl, 20 mM MgCl₂ at pH7.5. One portion of the cleavage products was denatured with glyoxal and analyzed in a 1% agarose gel to determine the extent of cleavage of the top strand or sense strand of the DNA substrate at the E2/E3 junction. Another portion of the nucleic acid cleavage products was analyzed in a denaturing 6% polyacrylamide gel to determine the extent of cleavage of both strands of the double stranded DNA substrate. The gels were dried and autoradiographed or quantitated by phosphorimaging with a Molecular Dynamics Phosphorimager 445.

The results indicated that the nucleotide integrase comprising an excised *al2* intron RNA from wild-type yeast bound to an *al2* intron-encoded protein from wild-type yeast cleaved the top strand of a substrate having the wt target sequence at the position marked by the arrowhead in Figure 3. The results also indicated that the group II intron RNA is integrated into the cleavage site of the sense strand. The results also indicated that the nucleotide integrase cleaved the bottom strand or antisense strand of the double-stranded DNA substrate at a location 10 base pairs downstream from the cleavage site in the first strand.

0.025 O.D.₂₆₀ units of the RNP particles of formulation 1 were reacted with six different derivatives of the wt DNA substrate of Figure 3. Each of the derivatives contained a single point mutation in IBS2 of the wt sequence shown in Figure 3. In the derivatives, the nucleotides in the -7, -8, -9, -10, -11, -12, and -13 were each changed to its complement. The reactions were conducted as and the cleavage products assayed on a 1% agarose gel

as described above. The results indicated that the ability of this nucleotide integrase to cleave a double-stranded DNA substrate was considerably reduced unless there was full complementarity between each of the nucleotides of EBS2 of the aI2 intron RNA and each of the nucleotides of the IBS2 of the substrate. The only exception occurred with the substrate having a mutation at the nucleotide at +7.

5 0.025 O.D.₂₆₀ units of the nucleotide integrase of formulation 1 were reacted with derivatives of the wt DNA substrate of Figure 3 in which the nucleotides at each of the positions from -14 to -21 in the wt sequence were separately changed to a mixture of the incorrect nucleotides. Thus, the nucleotide integrase was reacted with 10 different substrates, each of which contained a mixture of three mutations at a single site. The reactions were conducted as described above in example 1 and the cleavage products were glyoxylated and assayed on a 1% agarose gel. The results indicated that the nucleotide integrase cleaved substrates having point mutations at position -21, -20, -17, and -14 in the target sequence at levels that ranged from 67% to 115% of the levels achieved when the nucleotide integrase was reacted with the wt sequence depicted in Figure 3. The levels of cleavage were reduced to the greatest extent with the substrates having point mutations at -15 and -18. The level of cleavage that occurred with substrates having mutations at -15 and -18 was 9% and 3% of the cleavage obtained when the nucleotide integrase was reacted with the wt sequence depicted in Figure 3. Mutations at positions -16 and -19 had moderate effects, and substrates containing these mutation were cleaved by the nucleotide integrase at levels that were 23% and 31% of the levels achieved with a substrate having the wt sequence.

20 0.025 O.D.₂₆₀ units of the nucleotide integrase of formulation 1 were reacted with derivatives of the DNA substrate of Figure 3 in which the nucleotides at each of the positions from +1 to +10 in the wt sequence were separately changed to a mixture of three different bases. Thus, the nucleotide integrase was reacted with 30 different substrates, each of which had a mixture of the three different nucleotides. The reactions were conducted as described above in example 1 and the cleavage products were assayed on a 6% polyacrylamide gel to determine whether the nucleotides at these positions are required for cleavage of the antisense strand of the substrate containing the wt sequence. The cleavage products were also glyoxylated and analyzed on a 1% agarose gel to determine if changes in the nucleotides at these positions had any effect on the ability of the nucleotide integrase to cleave the top strand of the substrate. The results indicated that the aI2 nucleotide integrase cleaved substrates for the second strand having changes at position +1, +4, and +6 at levels that were 39, 33, and 29 %, respectively of the levels achieved when the nucleotide integrase was reacted with the wt sequence depicted in Figure 3. Changes in the nucleotides at the other positions, i.e., +2, +3, +5, +7, +8, +9, and +10 had little effect on the ability of the nucleotide sequence to cleave the second strand of the substrate. The results also indicated that changes in the nucleotides at each of these positions had little effect on the ability of the nucleotide integrase to cleave the top strand of the mutated substrate.

Comparative Example A

35 0.025 O.D.₂₆₀ units of the RNP particle preparations of formulations 1, 2, 4, 5 were reacted for 20 minutes with 125 fmoles (150,000 cpm) an internally-labeled DNA substrate having the wt sequence depicted in Figure 3. To verify cleavage, the products were glyoxylated and analyzed in a 1% agarose gel. The results indicated

that nucleotide integrases which lack excised $\alpha 2$ intron RNA or in which the intron-encoded protein lacks the nonconserved portion of the Zn domain, will neither cleave the double-stranded DNA substrate nor attach an RNA.

Example 2 Cleaving a Double-stranded DNA substrate with the Reconstituted RNP Particle Preparation of Formulation 12

The reconstituted RNP particle preparation of formulation 12 was reacted with 250 fmoles (300,000 cpm) of the 142 base pair DNA substrates generated from pE2E3 and which were 5' end-labeled on either the sense strand or the antisense strand for 20 minutes at 37°C. To verify cleavage of both strands of the substrate, the reaction products were extracted with phenol-CIA in the presence of 0.3 M NaOAc and 2 μ g single-stranded salmon sperm DNA followed by precipitation with ethanol. Reactions products were analyzed in a 6% polyacrylamide/8 M urea gel. The results indicated that the reconstituted particle preparation cleaves both strands of a double-stranded DNA substrate which contains the wild-type sequence shown in Figure 4. Similar results, i.e. cleavage of both strands, were obtained when the 5' end labeled substrates were incubated with the RNP particle preparation of formulation 10.

Example 3 Cleaving Double-stranded DNA Substrates with a Nucleotide Integrase Comprising a Modified $\alpha 2$ Intron RNA and an $\alpha 2$ -Encoded Protein.

0.025 O.D.₂₆₀ units of the RNP particles of formulation 13 in which the EBS1 of the $\alpha 2$ group II intron RNA was changed to the EBS1 sequence of the $\alpha 1$ intron RNA was reacted with the wt DNA substrate of Figure 3 and with a derivative thereof in which the nucleotides at position -1 to -6 were simultaneously changed to 5'TTAATG, which is the IBS1 sequence of the wt sequence for the $\alpha 1$ nucleotide integrase. The reactions were conducted and the cleavage products analyzed as described in example 1. The results indicated that an $\alpha 2$ nucleotide integrase comprising a group II intron RNA with a modified EBS1 was not able to cleave a substrate with the wt sequence but was able to cleave a substrate in which the nucleotides at position -1 to -6 were complementary to the modified EBS1.

Example 4 Cleaving Substrate with a Nucleotide Integrase Comprising a Wild-type or Modified $\alpha 2$ Intron RNA and an $\alpha 2$ -Encoded Protein

0.025 O.D.₂₆₀ units of the RNP particles of formulation 1 were reacted with three different derivatives of the DNA wt substrate of Figure 3. Each of the derivatives contained a single point mutation. In the derivatives the nucleotide at +1 was changed to either a C, G, or A. The derivatives were also reacted with a nucleotide integrase comprising an $\alpha 2$ intron RNA in which the nucleotide immediately preceding EBS1 was either an A, G, C, or T. The reactions were conducted and the cleavage products assayed on a 1% agarose gel as described in example 1. The results indicated that cleavage of the top strand is enhanced when the nucleotide at +1 is complementary to the nucleotide immediately preceding the EBS1 in the $\alpha 2$ intron RNA and that cleavage of the sense strand is strongly reduced when the target sequence has a G at the +1 position and the intron RNA has a purine nucleotide (A or G) at the δ position.

Example 5 Cleaving Double-Stranded DNA Substrates with a Nucleotide Integrase Comprising an aII intron RNA and an aII intron-encoded protein.

Double-stranded DNA substrates comprising either the wt sequence or an altered sequence having one of the eleven single point mutations depicted in Figure 6 were reacted with the RNP particle preparation of Formulation 3. For each reaction, 1.5 nM (150000 cpm) of a double-stranded DNA substrate was mixed with 0.025 OD₂₆₀ units of the RNP particle preparation in 10 µl of 50 mM Tris pH 7.5, 5 mM KCl, 10 mM MgCl₂, 5 mM DTT. The reaction mixtures were incubated for 20 minutes at 37°C. The reaction was stopped by adding 70 µl of 28.6 mM EDTA, 0.15 mg/ml tRNA. The nucleic acids were phenol extracted, ethanol precipitated, glyoxylated and analyzed on a 1% agarose gel.

The results indicated that the nucleotide integrase of formulation 3 cleaved substrate DNAs having mutations at positions -23, -20, -17, -16, -15 and -14 as efficiently as a substrate having the wt sequence depicted in Figure 6. Mutations at positions G(-22), G(-21), A(-19) and A(-18) reduced the efficiency of the cleavage somewhat from 75 to 25% of the cleavage that occurred with the wt sequence. The most critical nucleotide appears to be the C at position(-13). Mutations at this position reduced cleavage of the substrate to less than 1% of that which occurred with the wt sequence.

Example 6 Cleaving substrates with a Nucleotide Integrase Comprising an Ll.ltrB intron RNA and an Ll.ltrB intron-encoded protein.

Double-stranded DNA substrates comprising either the wt sequence or an altered wt sequence having one of the eleven single point mutations depicted in Figure 8 were reacted with the RNP particle preparation of Formulation 15. The point mutations occur at positions -23 to -13 in the wt sequence. For each reaction, 1.5 nM of a double-stranded DNA substrate was mixed with 0.025 OD₂₆₀ units of the RNP particle preparation in 10 µl of 50 mM Tris pH 7.5, 10 mM KCl, 10 mM MgCl₂, 5 mM DTT. The reaction mixtures were incubated for 20 minutes at 37°C. The reaction was stopped by adding 70 µl of 28.6 mM EDTA, 0.15 mg/ml tRNA. The nucleic acids were phenol extracted, ethanol precipitated, glyoxylated and analyzed on a 1% agarose gel.

The results indicated that the nucleotide integrase of formulation 15 cleaved substrate DNAs having mutations at positions C(-22), C(-18), and A(-14) at levels that were approximately 80% of the levels achieved with a substrate having the wt sequence depicted in Figure 8. Substrates having point mutations at positions G(-21), A(-20), T(-19) were cleaved at levels that were approximately 40% or less of the levels achieved using substrates having a wt sequence.

Example 7 Cleaving a Double-Stranded DNA Substrate with Purified RNP Particles

125 fmoles (150,000 cpm) of an internally-labeled substrate containing of yeast mitochondrial COX1 exons 2 and 3 (E2E3) and comprising the WT sequence shown in Figure 3 were incubated with 10 µl of each of the fractions obtained from the sucrose gradient in formulation 1a. Taking into account the composition of the fractions, the final reaction medium of 20 µl contained 100 mM KCl, 20 mM MgCl₂, 50 mM Tris-HCl, pH 7.5, and 5 mM DTT. Following a 20 minute reaction at 37°C, 30 µl of water, 5 µl 0.3 M NaOAc and 5 µg tRNA were added to the

fractions. The reaction products were phenol extracted, ethanol precipitated, glyoxalated, separated on a 1% agarose gel and analyzed by autoradiography of the dried gel. The results indicated that the purified RNP particles of formulation 1a are useful to cleave both strands of a double-stranded DNA substrate and to insert the aI2 intron RNA into the cleavage site.

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Example 8 Cleaving Both Strands of a Double-stranded DNA Substrate and Attaching a cDNA to the Cleavage Site of the Antisense Strand.

0.025 O.D.₂₆₀ units of the RNP particles from formulations 1,2,4,5,6,7,8,9, were incubated with 250 fmoles (300,000 cpm) of a 142 base pair DNA substrate comprising the WT sequence shown in Figure 3. DNA incubation products were analyzed in a 6% polyacrylamide/8 M urea gel.

A radiolabeled band corresponding to the 5' fragment was detected when RNP particles of formulations 1 and 2 were incubated with substrates that had been labeled on the 5' end of either the top strand or the bottom strand of the DNA substrate, indicating that these particles cleaved both strands of the DNA substrate. The RNP particles of formulation 1 cleaved the top strand precisely at the exon 2-exon 3 junction. The RNP particles of formulations 1 and 2 cleaved the bottom or antisense strand 10 base pairs downstream from the top or sense strand cleavage site. RNP particles of formulation 1 that had been treated with protease K, or RNase A, or boiled did not cleave either strand.

Radiolabeled bands were also detected when the RNP particles of formulation 4 were incubated with DNA substrates that had been 5' end-labeled on either the sense strand or antisense strand, indicating that this nucleotide integrase cleaved both strands of DNA substrate. The RNP particles of formulation 4 contain a modified, excised aI2 RNA and an aI2-encoded protein which lacks detectable reverse transcriptase activity. Although the extent of cleavage of RNP particles of formulation 4 is somewhat reduced compared to cleavage with the RNP particle preparation of formulation 1, the endonuclease activity of the RNA is present even when the reverse transcriptase activity of the aI2-encoded protein is absent.

The radiolabeled bands were detected when the RNP particles of formulation 5 were incubated with the DNA substrate that had been labeled on the 5' end of either the top or bottom strand. In quantitative assays normalized by either O.D.₂₆₀ or soluble aI2 reverse transcriptase activity, the cleavage activities for the top and bottom strands by the RNP particles of formulation 5 were 6% and 25%, respectively, of activities of the RNP particles of formulation 1.

A radiolabeled band corresponding to the 5' fragment was detected when the DNA substrate labeled on the 5' end of the top strand was incubated with the RNP particles of formulation 6, but a band corresponding to the 5' fragment of the top strand was not detected when the RNP particles of formulation 6 were incubated with a DNA substrate that had been labeled on the 5' end of the bottom strand. The RNP particles of formulation 6 contain a modified, excised aI2 intron RNA and an aI2-encoded protein that has an alteration in one of the putative endonuclease motifs. Similar results were obtained with the RNP particles of formulation 7, which contains a modified, excised aI2 intron RNA and an aI2-encoded protein in which the conserved portion of the Zn domain is

absent. Likewise, RNP particles of formulations 8 and 9, each of which contains a modified, excised aI2 intron RNA and an aI2-encoded protein in which there is a mutation in the Zn²⁺-like motif, cleaved the sense strand but not the antisense strand of the DNA substrate. For the RNP particles of formulations 6, 7, 8, and 9, the level of sense-strand cleavage was proportional to the amount of RNA-DNA products detected in the agarose gels. These findings indicate that the antisense strand endonuclease activity of the aI2-encoded protein is associated with the Zn domain.

A radiolabeled band corresponding to the 5' fragment was detected when the reconstituted RNP particle preparation of formulation 12 was incubated with substrates that had been labeled on the 5' end of either the sense strand or the antisense strand of the DNA substrate. These results establish that the reconstituted RNP particle preparation cleaves both strands of the DNA substrate.

Thus, both the catalytic RNA molecule of the nucleotide integrase and the intron-encoded protein are required for cleavage of both strands of the double stranded DNA. Certain modifications in the Zn domain and the X domain of intron-encoded protein disrupt the cleavage of the antisense strand of the nucleotide integrase

0.025 O.D.₂₆₀ units of the RNP particle preparations of formulations 1, 2, 4 and 5 were combined in 10 µl of reaction medium with 1 µg of plasmid containing the wild-type sequence depicted in Figure 4. The reaction medium contained 0.2 mM each of dATP, dGTP and dTTP, 10 µCi [α-³²P]-dCTP (3,000 Ci/mmol; DuPont NEN, Boston, MA), 100 mM KCl, and 5 mM dithiothreitol, 2 mM MgCl₂, and 50 mM Tris-HCl, pH 8.5. The reaction was initiated by addition of the RNP preparations, incubated for 10 minutes at 37°C, and chased with 0.2 mM dCTP for another 10 minutes. After the chase period, the reactions were terminated by extraction with phenol-CIA (phenol-chloroform-isoamyl alcohol; 25:24:1) in the presence of 0.3 M sodium acetate, pH 7.8, and 5 µg E. coli tRNA carrier (Sigma, St. Louis, MO). Products were ethanol precipitated twice and resolved in 1% agarose gels containing 90 mM Tris-borate, pH 8.3, 2 mM EDTA and 0.05% ethidium bromide. The results indicated that the RNP particles of formulations 1 and 2 catalyze the formation of a DNA molecule on the cleaved DNA substrate. The results also indicated that a nucleotide integrase which lacks an excised group II intron RNA or which contains a group II intron-encoded protein that lacks a reverse transcriptase domain does not catalyze the formation of a cDNA molecule on the cleaved strand.

Cleavage of single stranded DNA

An aI2 nucleotide integrase comprising an excised aI2 RNA and aI2-encoded protein was used to cleave a single stranded DNA comprising an IBS2 and IBS1 sequence complementary to the EBS1 and EBS2 sequences of the wild-type aI2 intron RNA. The reaction is greatly improved when the 3 nucleotides +1 to +3 can base-pair with the 3 nucleotides immediately upstream of EBS1. The most preferred reaction conditions for cleavage of the substrate and insertion of the intron RNA into the cleavage site by the nucleotide integrase, are 100 mM KCl, 20 mM MgCl₂, pH 7.5, 5 mM DTT and 37°C.

CLAIMS

What is claimed is:

1. A method of cleaving a double stranded DNA substrate at a cleavage site, said substrate having a recognition site, said method comprising the following steps:

(a) providing a nucleotide integrase comprising;

(i) a group II intron RNA having a first hybridization sequence capable of hybridizing with a first intron RNA binding sequence of one strand of the DNA substrate and a second hybridization sequence capable of hybridizing with a second RNA binding sequence on said one strand of the substrate; and

(ii) a group II intron-encoded protein capable of binding with at least one nucleotide in a first sequence element in the recognition site of the substrate, said group II intron-encoded protein being bound to said group II intron RNA; and
(b) reacting the nucleotide integrase with the substrate to permit the nucleotide integrase

to cleave said one strand of the DNA substrate and to insert the group II intron RNA into the cleavage site.

2. The method of claim 1 wherein there is at least 80% complementarity between the first hybridization sequence and the first intron RNA binding sequence and at least 80% complementarity between the second hybridization sequence and the second intron RNA-binding sequence.

3. The method of claim 1 wherein the group II intron RNA further comprises a δ nucleotide that is complementary to a δ' nucleotide on said one strand of the substrate, said δ' nucleotide being located at position +1 relative to the cleavage site.

4. The method of claim 1 wherein the group II intron RNA is a wild-type or modified α 12 intron RNA and wherein the group II intron-encoded protein is an α 12 intron-encoded protein

5. The method of claim 4 wherein said one strand of the substrate comprises a T at position -13 relative to the cleavage site, a T at position -15 relative to the cleavage site, a C at position -18 relative to the cleavage site, and a G at position -16 or position -19 relative to the cleavage site.

6. The method of claim 4 wherein said one strand of said substrate comprises a G at -19, a C at -18, a G at -16, a T at -15, and a T at -13 relative to the cleavage site.

7. The method of claim 1 wherein the group II intron RNA is a wild-type or modified α 11 intron RNA and wherein said group II intron-encoded protein is a protein encoded by an α 11 intron.

8. The method of claim 7 wherein said one strand of the substrate has a C at -13 relative to the cleavage site.

9. The method of claim 7 wherein said one strand of the substrate comprises a G at -22, a G at -21, an A at -19, an A at -18, and a C at -13 relative to the cleavage site.

10. The method of claim 1 wherein the nucleotide integrase comprises a wild-type or modified L1.ltrB intron RNA and a protein encoded by the L1.ltrB intron.

- 1 11. The method of claim 10 wherein said one strand of the substrate comprises a G at -21 and an A at -20 relative to
2 the cleavage site.
- 1 12. The method of claim 11 wherein said one strand of the substrate comprises a G at -21, an A at -20, a T at -19,
2 a G at -17 and a G at -15 relative to the cleavage site.
- 1 13. A method of cleaving a single-stranded nucleic acid substrate at a cleavage site comprising the following steps:
2 (a) providing a nucleotide integrase comprising;
3 (i) a group II intron RNA having a first hybridizing sequence
4 capable of hybridizing with a first intron RNA binding sequence on the nucleic
5 acid substrate and a second hybridizing sequence capable of hybridizing with a second
6 intron RNA binding sequence on said nucleic acid substrate, and
7 (ii) a group II intron-encoded protein bound to said group II intron RNA; and
8 (b) reacting the nucleotide integrase with the substrate to permit the nucleotide integrase to cleave
9 the nucleic acid substrate and to insert the group II intron RNA into the cleavage site.
- 1 14. The method of claim 13 wherein the substrate is RNA.
- 1 15. The method of claim 13 wherein the substrate is DNA.
- 1 16. The method of claim 13 wherein the nucleotide integrase is selected from a group consisting of:
2 (a) a wild-type or modified $\alpha 2$ intron RNA and an $\alpha 2$ intron-encoded protein;
3 (b) a wild-type or modified $\alpha 1$ intron RNA and an $\alpha 1$ intron-encoded protein; and
4 (c) a wild-type or modified L1.ltrB intron RNA and an L1.ltrB intron-encoded protein.
- 1 17. A method of cleaving both strands of a double-stranded DNA substrate comprising the following steps:
2 (a) providing a nucleotide integrase comprising;
3 (i) a group II intron RNA having a first hybridizing sequence capable of
4 hybridizing with a first intron RNA binding sequence on one strand of the DNA substrate
5 and a second hybridizing sequence capable of hybridizing with a second intron RNA
6 binding sequence on said one strand of the DNA substrate; and
7 (ii) a group II intron-encoded protein capable of binding to at least one nucleotide
8 in a first sequence element and to at least one nucleotide in a second sequence element
9 of the substrate, said group II intron-encoded protein being bound to said group II intron
10 RNA; and
11 (c) reacting the nucleotide integrase with the substrate for a time and at a temperature
12 sufficient to permit the nucleotide integrase to cleave both strands of the DNA
13 substrate and to insert the group II intron RNA into the cleavage site on said one strand.
- 1 18. The method of claim 17 wherein the nucleotide integrase is selected from a group consisting of:

- 2 (a) a wild-type or modified aI2 intron RNA and an aI2 intron-encoded protein;
- 3 (b) a wild-type or modified aI1 intron RNA and an aI1 intron-encoded protein; and
- 4 (c) a wild-type or modified L1tr.B intron RNA and an L1.ltrB intron-encoded protein.

1 19. The method of claim 17 wherein there is at least 80% complementarity between the first hybridization sequence
2 and the first intron RNA binding sequence and wherein there is at least 80% complementarity between the second
3 hybridization sequence and the second intron RNA binding sequence.

1 20. The method of claim 17 wherein the group II intron RNA is a wild-type or modified aI2 intron RNA, wherein
2 the group II intron-encoded protein is an aI2 intron-encoded protein; and wherein said one strand of the substrate
3 comprises a C at -18, a T at -15, a T at -13, a G at -13 or -16, a T at +1, a T at +4 and a G at +4.

1 21. The method of claim 17 wherein the group II intron RNA is a wild-type or modified aI1 intron RNA: wherein
2 the group II intron-encoded protein is a protein encoded by an aI1 intron; and wherein said one strand of the
3 substrate comprises a C at -13, a T at +1, a T at +2, a T at +3, a T at +4, an A at +5, a G at +6, a T at +7, and an A at
4 +8.

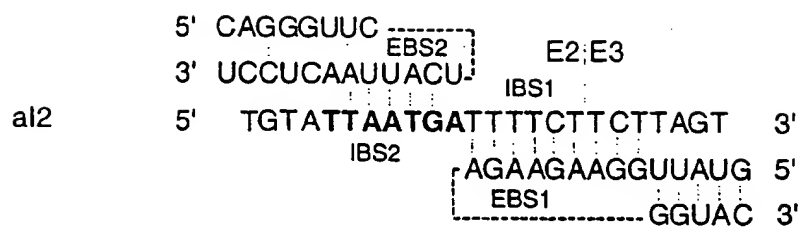
1 22. The method of claim 17 wherein the group II intron RNA is a wild-type or modified L1.ltrB intron RNA and the
2 group II intron-encoded protein is a protein encoded by an L1.ltrB intron; and wherein the top strand of the
3 substrate comprises a G at -21, an A at -20, a C at +1, an A at +2, a T at +3, an A at +4, a T at +5, a C at +6, an A at
4 +7, and a T at +8.

1 23. The method of claim 17 wherein the group II intron encoded protein comprises a reverse transcriptase domain,
2 and wherein the nucleotide integrase and the substrate are reacted in a reaction mixture comprising dATP, dGTP,
3 dTTP, and dCTP such that a cDNA molecule is formed in the cleavage site on the other strand of the DNA
4 substrate.

1 24. A method of detecting the presence of a nucleotide recognition site in a nucleic acid substrate comprising the
2 steps of:

- 3 (a) providing a nucleotide integrase capable of cleaving a nucleic acid substrate having a
4 recognition site;
- 5 (b) reacting the nucleic acid substrate with said nucleotide integrase; and
- 6 (c) assaying for cleavage of the nucleic acid substrate, wherein cleavage is indicative of
7 the presence of the recognition site in the nucleic acid substrate.

FIGURE 1



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FIGURE 2

S.cerevisiae 161 mt cox1 E1, a11, E2, a12, E3

```
>E1
ATGGTACAAA GATGATTATA TTCAACAAAT GCAAAAGATA TTGCAGTATT ATATTTTATG      60
TTAGCTATTT TTAGTGGTAT GGCAGGAACA GCAATGTCTT TAATCATTAG ATTAGAATTA      120
GCTGCACCTG GTTCACAATA TTTACATGGT AATTCACAGT TATTTAATGG TCGGCCTCTC      180
AGTGCGTATA TTTCGTTGAT GCGTCTAGCA TTAGTATTAT GAATCATCAA TAGATACTTA      240
AAACATATGA CTAACTCAGT AGGGGCTAAC TTTACGGGGA CAATAGCATG TCATAAAACA      300
CCTATGATTA GTGTAGGTGG AGTTAAGTGT TACATGGTTA GGTTAACGAA CTTCTTACAA      360
> EBS2<
GTCTTATCA GGATTACAAT TTCTCTTAT CATTTGGATA TAGTAAAACA AGTTTGATTA      420
> EBS1<
TTTTACGTTG AGGTAATCAG ATTATGATTC ATTGTTTTAG ATAGCACAGG CAGTGTGAAA      480
AAGATGAAGG ACCTAAATAA CACAAAAGGA AATACGAAAA GTGAGGGATC AACTGAAAGA      540
GGAAACTCTT GAGTTGACAG AGGTATAGTA GTACCGAATA CTCAAATAAA AATGAGATTT      600
TTAAATCAAG TTAGATACTA TTCAGTAAAT AATAATTAA AAATAGGGAA GGATACCAAT      660
ATTGAGTTAT CAAAAGATAC AAGTACTTCG GACTTGTTAG AATTTGAGAA ATTAGTAATA      720
GATAATATAA ATGAGGAAAA TATAAATAAT AATTTATTAA GTATTATAAA AAACGTAGAT      780
ATATTAATAT TAGCATATAA TAGAATTAAG AGTAAACCTG GTAATATAAC TCCAGGTACA      840
ACATTAGAAA CATTAGATGG TATAAATATA ATATATTAA ATAAATTATC AATGAATTA      900
GGAACAGGTA AATTCAAATT TAAACCATG AGAATAGTTA ATATTCCTAA ACCTAAAGGT      960
GGTATAAGAC CTTTAAGTGT AGGTAATCCA AGAGATAAAA TTGTACAAGA AGTTATAAGA     1020
```

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FIGURE 2 continued

ATAATTTTAG ATACAATTTT TGATAAAAAG ATATCAACAC ATTACATGG TTTTAGAAAG 1080
AATATAAGTT GTCAAACAGC AATTGAGAA GTTAGAAATA TATTGGTGG AAGTAATTGA 1140
TTTATTGAAG TAGACTTAAA AAAATGTTTT GATACAATTT CTCATGATT AATTATTAAA 1200
GAATTAAAAA GATATATTTT AGATAAAGGT TTTATTGATT TAGTATATAA ATTATTAAGA 1260
GCTGGTTATA TTGATGAGAA AGGAACTTAT CATAAACCTA TATTAGGTTT ACCTCAAGGA 1320
TCATTAAFTA GTCTATCTT ATGTAATATT GTAATAACAT TGGTAGATAA TTGATTAGAA 1380
GATTATATTA ATTATATATA TAAAGGTAAA GTTAAAAAC AACATCTAC ATATAAAAAA 1440
TTATCAAGAA TAATTGCAA AGCTAAAATA TTTTCGACAA GATTAAAATT ACATAAGAA 1500
AGAGCTAAAG GCCCACTATT TATTTATAAT GATCCTAATT TCAAGAGAAT AAAATACGTT 1560
AGATATGCAG ATGATATTTT AATTGGGGTA TTAGGTTCAA AAAATGATTG TAAAATAATC 1620
AAAAGAGATT TAAACAATTT TTTAAATTCA TTAGGTTTAA CTATAAATGA AGAAAAACT 1680
TTAATTACTT GTGCAACTGA ACTACCAGCA AGATTTTTAG GTTATAATAT TTCAATTACA 1740
CCTTTAAAAA GAATACCTAC AGTTACTAAA CTAATTAGAG GTAACTTAT TAGAAGTAGA 1800
AATACAATA GACCTATTAT TAATGCACCA ATTAGAGATA TTATCAATAA ATTAGCTACT 1860
AATGGATATT GTAAGCATAA TAAAAATGGT AGAATAGGAG TGCCTACAAG AGTAGGTAGA 1920
TGACTATATC AAGAACCTAG AACAATTATT AATAATTATA AAGCGTTAGG TAGAGGTATC 1980
TTAAATTATT ATAAATTAGC TACTAATTAT AAAAGATTAA GAGAAAGAAT CTATTACGTA 2040
TTATATTATT CATGTGATT AACTTTAGCT AGTAAATATA GATTAAAAAC AATAAGTAAA 2100

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FIGURE 2 continued

2160
ACTATTAAAA AATTGTTA TAATTAAAT ATTATTGAAA ATGATAAATT AATTGCCAAT
2220
TTTCCAAGAA ATACTTTTGA TAATATCAAA AAAATTGAAA ATCATGGTAT ATTTATATAT
2280
ATATCAGAAG CTAAAGTAAC TGATCCTTTT GAATATATCG ATTCAATTAA ATATATATTA
2340
CCTACAGCTA AAGCTAATTT TAATAAACCT TGTAGTATTT GTAATTCAAC TATTGATGTA
2400
GAAATACATC ATGTTAAACA ATTACATAGA GGTATATTAA AAGCACTTAA AGATTATATT
2460
CTAGGTAGAA TAATTACCAT AAACAGAAAA CAAATTCAT TATGTAAACA ATGTCATATT
2520
AAAACACATA AAAATAAATT TAAAAATATA GGACCTGGTA TATAAAATCT ATTATTAATG
2580
ATACTCAATA TGGAAAGCCG TATGATGGGA AACTATCAGG TACGGTTTGG GAAAGGCTCT
aI1>E2
2640
TTAACACGTG GCAACATAGG TTAATTTGCT ATTTTCATTT TAGTAGTTGG TCATGCTGTA
E2>BI2
2700
TTAATGATTT TCTGTGCGCC GTTTCGCTTA ATTTATCACT GTATTGAAAT GTTAATTGAT
2760
AAACATATCT CTGTTTATTC AATTAATGAA AACTTTACCG TATCATTTTG GTTCTGATTA
2820
TTAGTAGTAA CATACATAGT ATTTAGATAC GTAAACCATA TGGCTTACCC AGTTGGGGCC
2880
AACTCAACGG GGACAATAGC ATGCCATAAA AGCGCTGGAG TAAACAGCC AGCGCAAGGT
>EBS2<
AAGAACTGTC CGATGGCTAG GTTAACGAAT TCCTGTAAAG AATGTTTAGG GTTCTCATT
>EBS1<
3000
ACTCCTTCCC ACTTGGGGAT TGTGATTCAT GCTTATGTAT TGGAGAAGA GGTACACGAG
3060
TTAACCAAAA ATGAATCATT AGCTTTAAGT AAAAGTTGAC ATTTGGAGGG CTGTACGAGT
3120
TCAAATGGAA AATTAAGAAA TACGGGATTG TCCGAAAGG GAAACCTGG GGATAACGGA
3180
GTCTTCATAG TACCCAAATT TAATTAAAT AAAGCGAGAT ACTTTAGTAC TTTATCTAAA

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FIGURE 2 continued

TTAAATGCAA GGAAGGAAGA CAGTTTAGCG TATTTAACAA AGATTAATAC TACGGATTTT 3240
TCCGAGTTAA ATAAATTAAT AGAAAATAAT CATAATAAAC TTGAAACCAT TAATACTAGA 3300
ATTTTAAAT TAATGTCAGA TATTAGAATG TTATTAATTG CTTATAATAA AATTAAAAGT 3360
AAGAAAGGTA ATATATCTAA AGGTTCTAAT AATATTACCT TAGATGGGAT TAATATTTCA 3420
TATTTAAATA AATTATCTAA AGATATTAAC ACTAATATGT TTAAATTTTC TCCGGTTAGA 3480
AGAGTTGAAA TTCCTAAAC ATCTGGAGGA TTTAGACCTT TAAGTGTGG AAATCCTAGA 3540
GAAAAAATG TACAAGAAAG TATGAGAATA ATATTAGAAA TTATCTATAA TAATAGTTTC 3600
TCTTATTATT CTCATGGATT TAGACCTAAC TTATCTTGT TAACAGCTAT TATTCAATGT 3660
AAAAATTATA TGCAATACTG TAATTGATTT ATTAAAGTAG ATTTAAATAA ATGCTTTGAT 3720
ACAATGCCAC ATAATATGTT AATTAATGTA TTAAATGAGA GAATCAAAGA TAAAGGTTTC 3780
ATAGACTTAT TATATAAATT ATTAAGAGCT GGATATGTTG ATAAAAATAA TAATTATCAT 3840
AATACRACCT TAGGAATTC TCAAGGTAGT GTTGTCAGTC CTATTTTATG TAATATTTTT 3900
TTAGATAAAT TAGATAAATA TTTAGAAAAT AAATTTGAGA ATGAATTCAA TACTGGAAAT 3960
ATGTCTAATA GAGGTAGAAA TCCAATTTAT AATAGTTTAT CATCTAAAT TTATAGATGT 4020
AAATTATTAT CTGAAAAATT AAAATTGATT AGATTAAGAG ACCATTACCA AAGAAATATG 4080
GGATCTGATA AAAGTTTAA AAGAGCTTAT TTTGTTAGAT ATGCTGATGA TATTATCATT 4140
GGTGTAAATGG GTTCTCATAA TGATTGTAAA AATATTTTAA ACGATATTAA TAACCTCTTA 4200
AAAGAAAAT TAGGTATGTC AATTAATATA GATAAATCCG TTATTAAACA TTCTAAAGAA 4260

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FIGURE 2 continued

GGAGTTAGTT TTTTAGGGTA TGATGTAAAA GTTACACCTT GAGAAAAAAG ACCTTATAGA 4320
ATGATTAAAA AAGGTGATAA TTTTATTAGG GTTAGACATC ATACTAGTTT AGTTGTTAAT 4380
GCCCCATTGA GAAGTATTGT AATAAAATTA AATAAACATG GCTATTGTTT TCATGGTATT 4440
TTAGGAAAAAC CCAGAGGGGT TGAAGATTA ATTCATGAAG AAATGAAAAAC CATTTTAAATG 4500
CATTACTTAG CTGTTGGTAG AGGTATTATA AACTATTATA GATTAGCTAC CAATTTTACC 4560
ACATTAAGAG GTAGAATTAC ATACATTTTA TTTTATTCAT GTTGTTTAAC ATTAGCAAGA 4620
AAATTTAAAT TAAATACTGT TAAGAAAGTT ATTTTAAAT TCGGTAAAGT ATTAGTTGAT 4680
CCTCATTCAA AAGTTAGTTT TAGTATTGAT GATTTTAAAA TTAGACATAA AATAAATATA 4740
ACTGATTCTA ATTATACACC TGATGAAATT TTAGATAGAT ATAAATATAT GTTACCTAGA 4800
TCTTTATCAT TATTAGTGG TATTGTCAA ATTTGTGGTT CTAAACATGA TTTAGAAGTA 4860
CATCACGTAA GAACATTAAA TAATGCTGCC AATAAAATTA AAGATGATTA TTTATTAGGT 4920
AGAATGATTA AGATAAATAG AAAACAAATT ACTATCTGTA AAACATGTCA TTTTAAAGTT 4980
CATCAAGGTA AATATAATGG TCCAGGTTTA TAATAATTAT TATACTCCTT CGGGGTCGCC 5040
GCGGGGGCGG GCGGACTAT TAAATATGCG TTAAATGGAG AGCCGTATGA TATGAAAGTA 5100
TCACGTACGG TTCGGAGAGG GCTCTTTTAT ATGAATGTTA TTACATTCAG ATAGGTTTGC 5160
aI2>E3 E3
TACTCTACTC TTAGTAATGC CTGCTTTAAT TGGAGGTTTT GGT

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Yeast a12 target site: protein recognition region

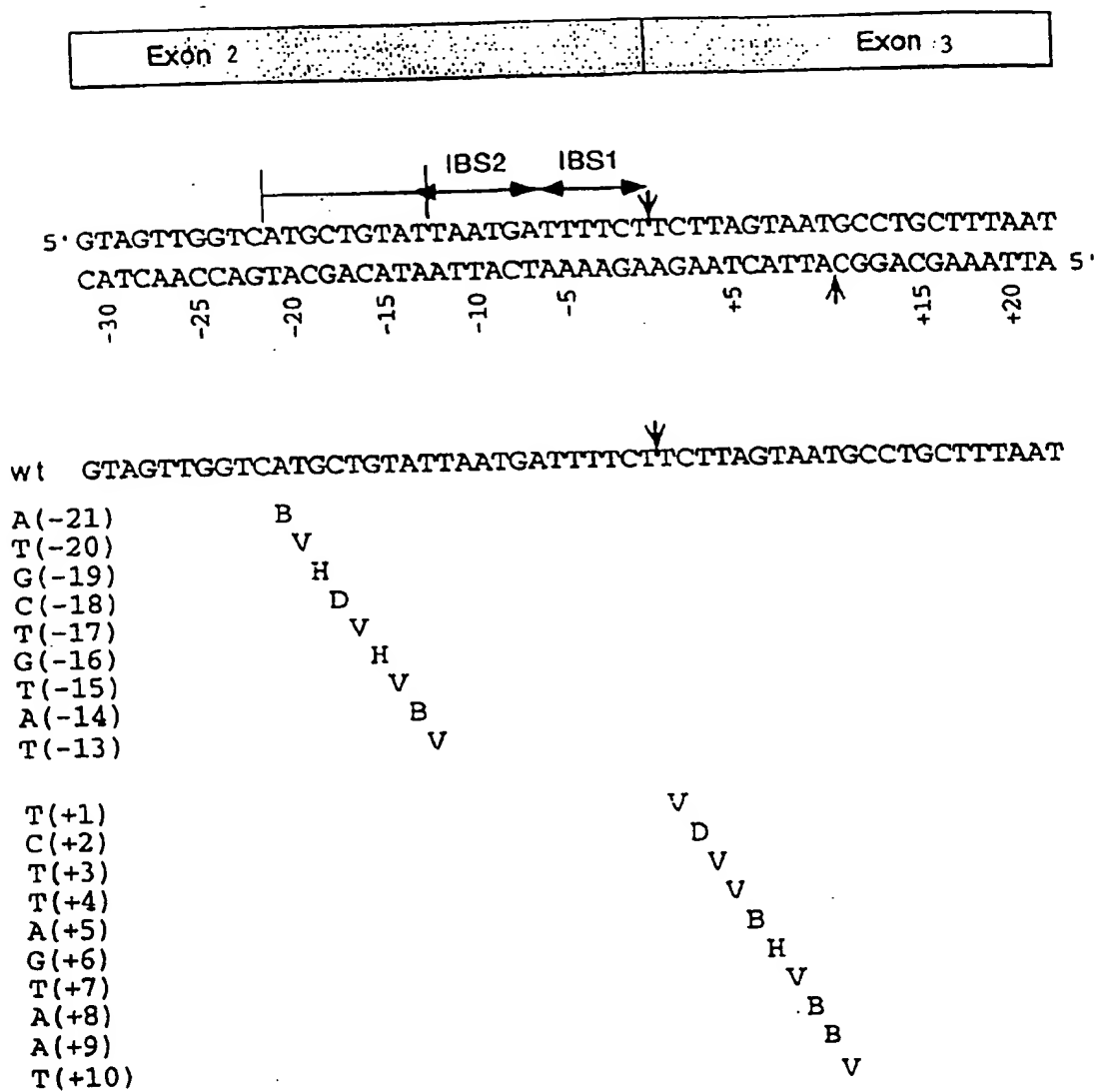


FIGURE 3

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Sequence requirements for recognition of target DNA by the *ai2*
nucleotide integrase

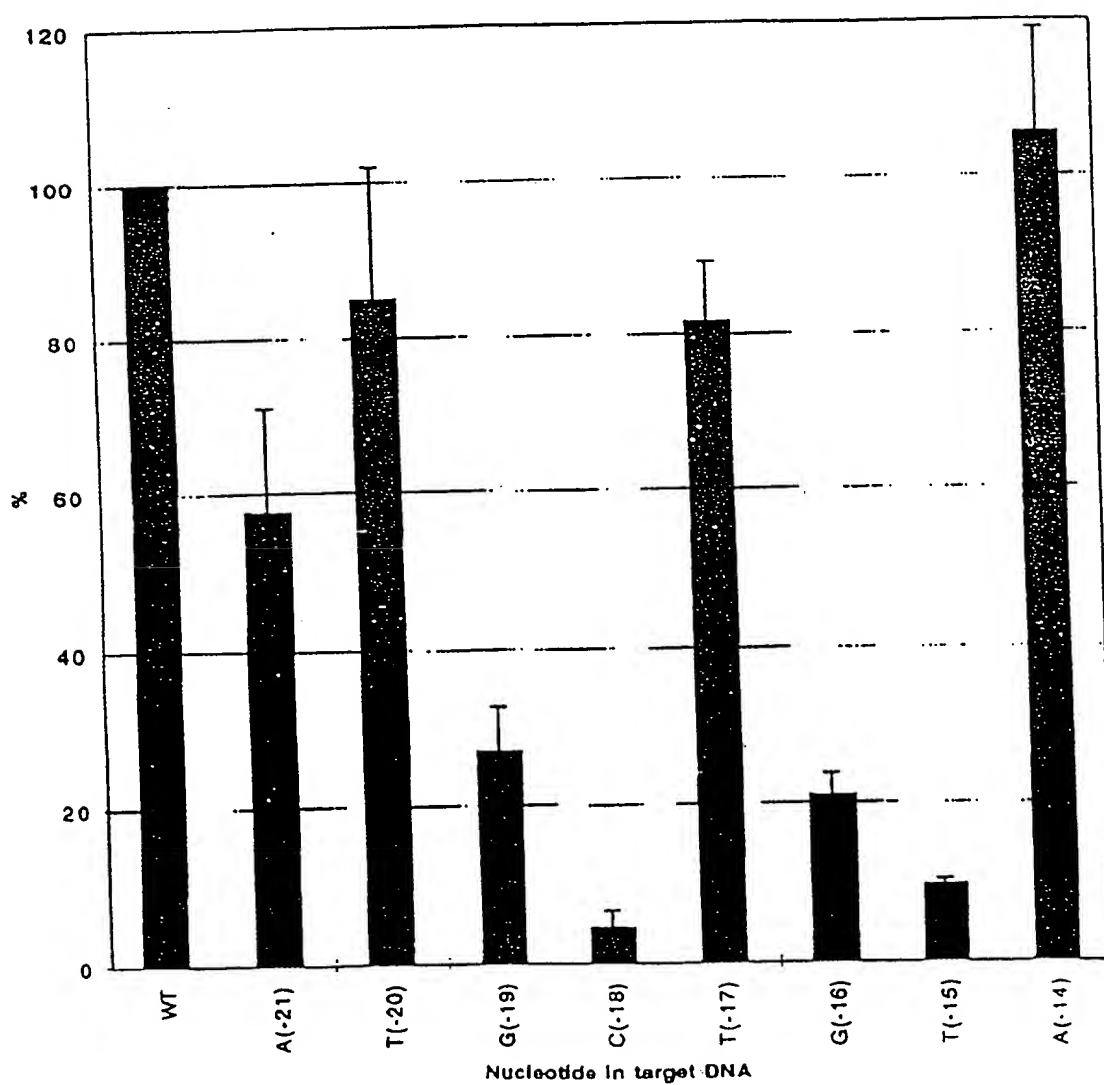


FIGURE 4

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Sequence requirements for bottom strand cleavage of target
DNA by the $\alpha 2$ nucleotide integrase

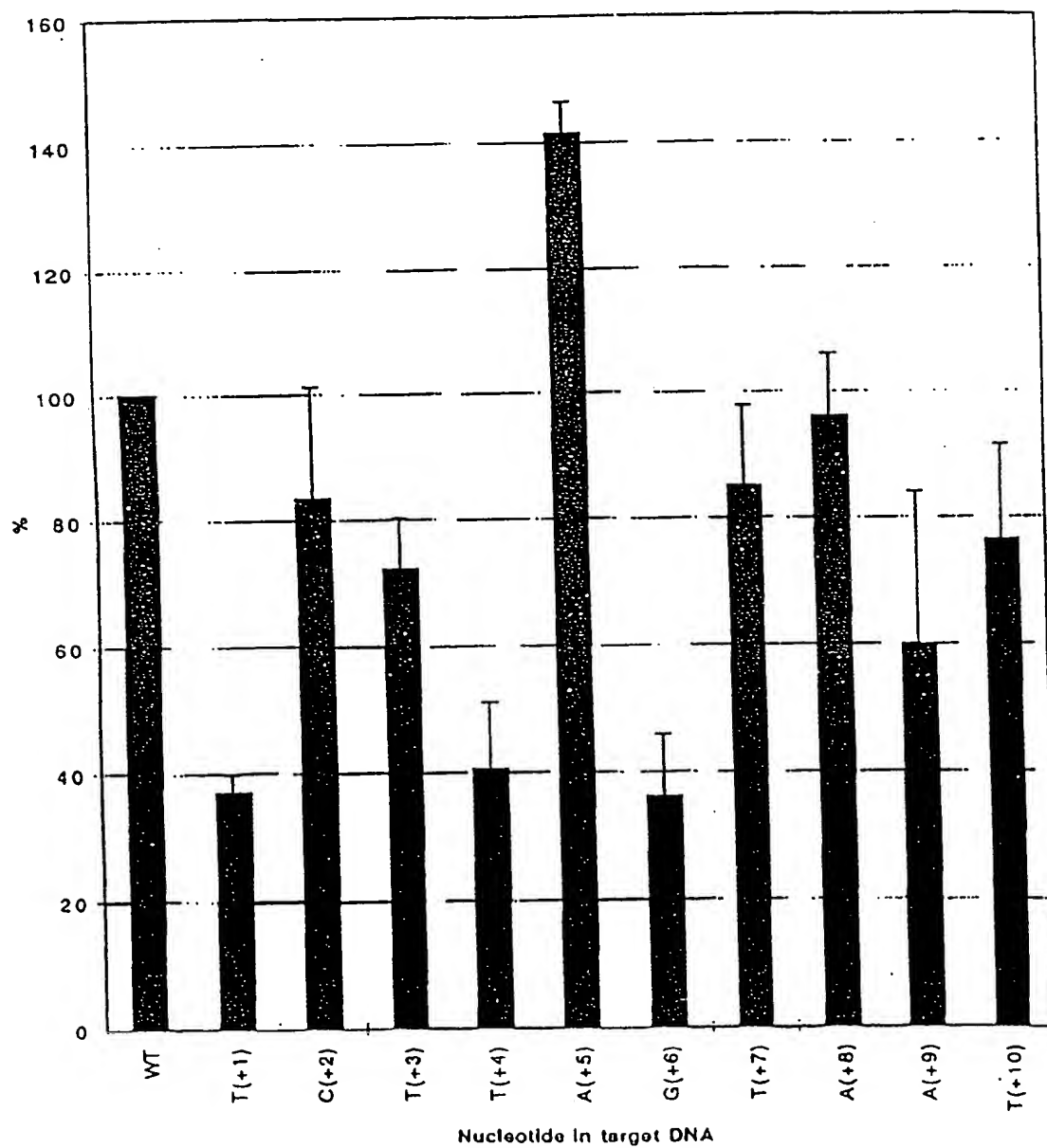
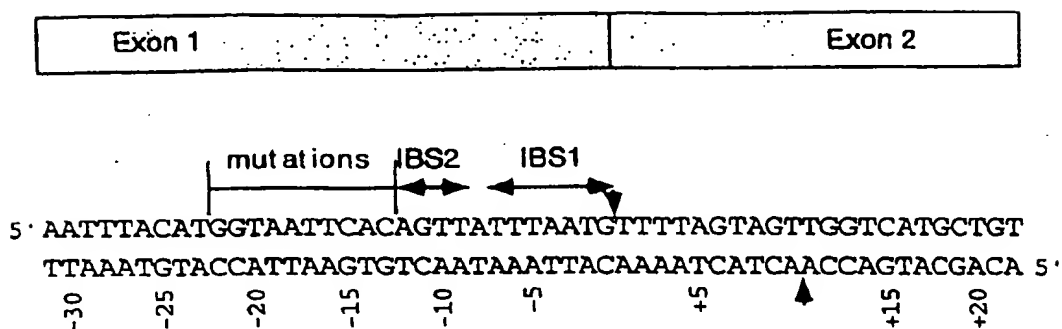


FIGURE 5

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Yeast a11 target site: protein recognition region



wt AATTTACATGGTAATTCACAGTTATTTAATG

T(-23) V
 G(-22) H
 G(-21) H
 T(-20) V
 A(-19) B
 A(-18) B
 T(-17) V
 T(-16) V
 C(-15) D
 A(-14) B
 C(-13) D

FIGURE 6

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Sequence requirements for recognition of target DNA by the *ai1*
nucleotide integrase

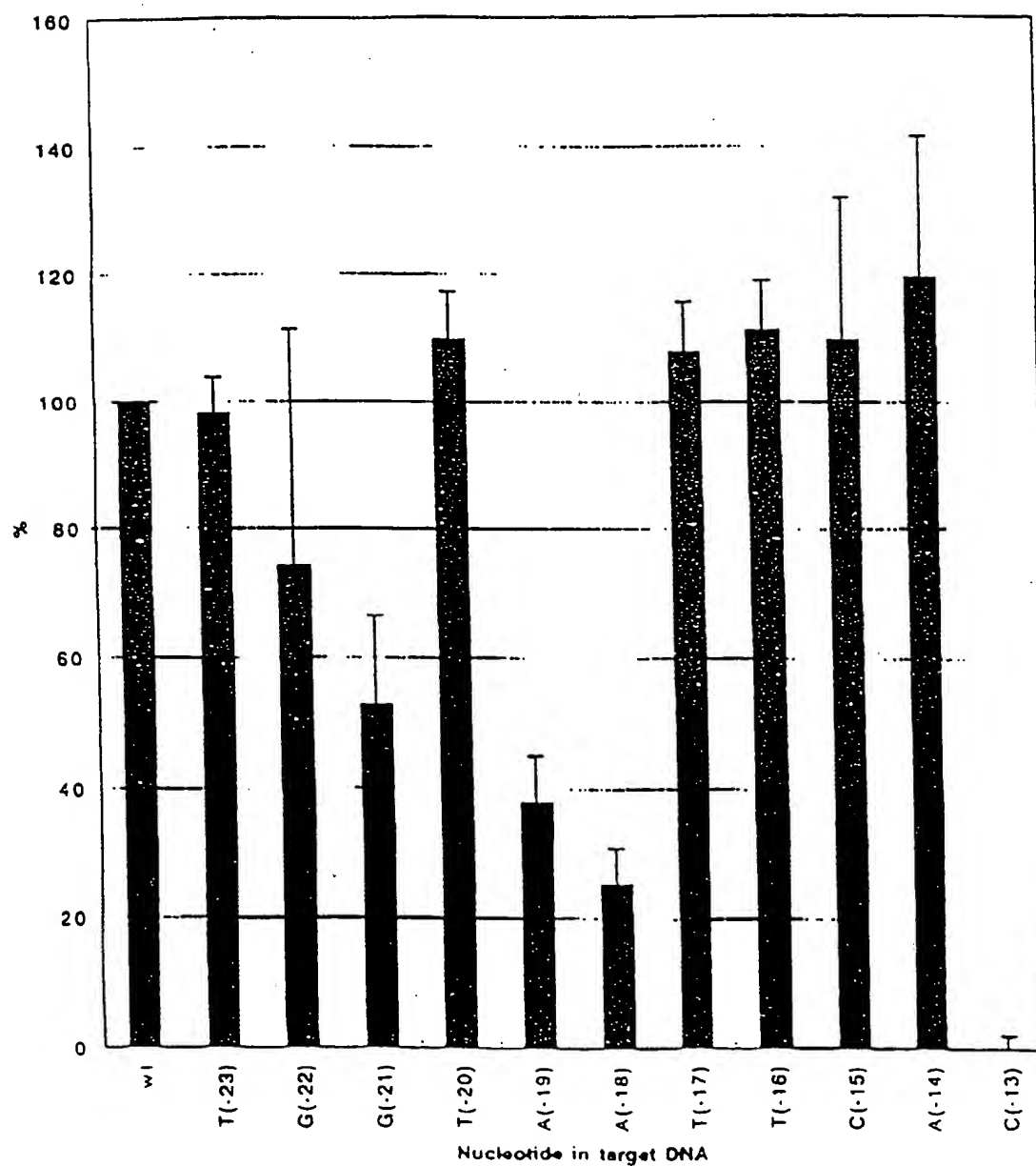
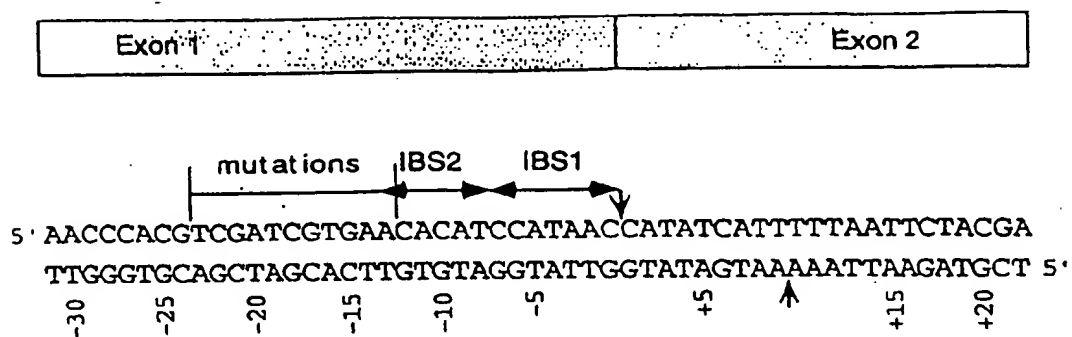


FIGURE 7

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Lactococcus ItrA target site: protein recognition region



WT	CCCACGTCGATCGTGAACACATCCATAACCATATCATTTTTAATTCTACGA
T(-23)	V
C(-22)	D
G(-21)	H
A(-20)	B
T(-19)	V
C(-18)	D
G(-17)	H
T(-16)	V
G(-15)	H
A(-14)	B
A(-13)	B
N	DHBVDHVBHBB
N-3	DHBVD BB
N-4	D D VBHBB
N-5	D D V BB

FIGURE 8

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Sequence requirements for recognition of target DNA by the
LtrA nucleotide integrase

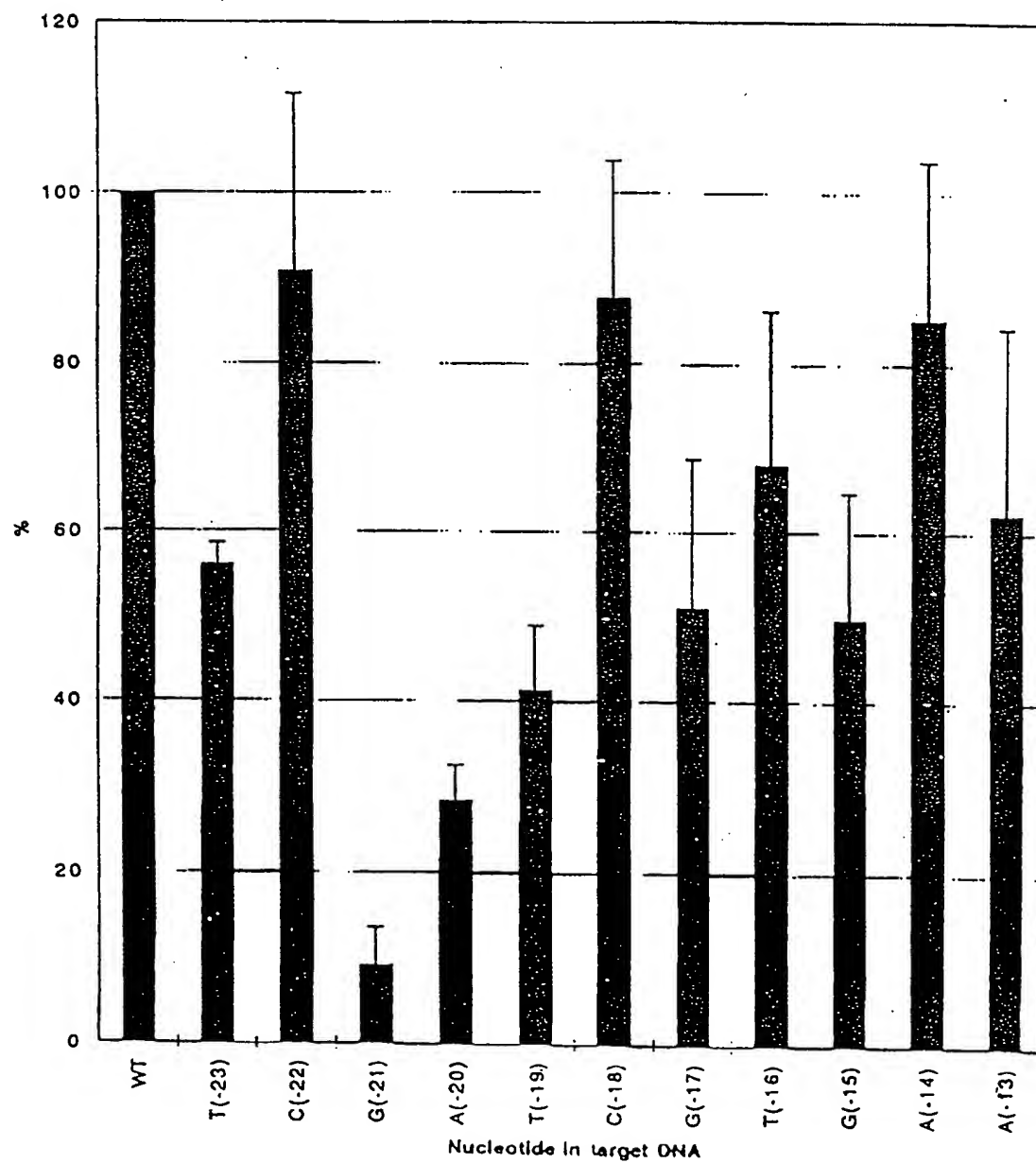


FIGURE 9

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LtrB-II.E3.fragment

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      10      20      30      40      50      60
AAGCTTAGAGAAAAATAATGCGGTGCTTGGTCATCACCTCATCCAATCATTTCCTCTGA
TTGCAATCTCTTTTATTACGCCACGAACCAAGTAGTGAGTAGGTTAGTAAAGAGGACT

      70      80      90      100     110     120
TGACAATCTAACTCCTGAACAAATTCATGAAATAGGTCGTCAAACCATATTAGAATTTAC
ACTGTTAGATTGAGGACTTGTTTAAGTACTTTATCCAGCAGTTTGGTATAATCTTAAATG

      130     140     150     160     170     180
AGGTGGCGAATATGAATTTGTGATTGCAACCCACGTCGATCGTGAACACATCCATAACT
TCCACCGCTTATACTTAAACACTAACGTTGGGTGCAGCTAGCACTTGTGTAGGTATTGCA

      190     200     210     220     230     240
CGCCCCAGATAGGGTGTAAAGTCAAAGTAGTTTAAAGTACTACTCTGTAAAGATAACACAGA
CGCGGGTCTATCCCAAAATTCAGTTCATCAAATTCATGATGAGACATTCTATTGTGTCT

      250     260     270     280     290     300
AAACAGCCAACCTAACCGAAAAGCGAAAGCTGATACGGGAACAGAGCACGGTTGGAAAGC
TTTGTGGGTGGATTGGCTTTTCGCTTTGACTATGCCCTTGCTCGTGCCAACCTTTTCG

      310     320     330     340     350     360
GATGAGTTACCTAAAGACAATCGGGTACGACTGAGTCGCAATGTTAATCAGATATAAGGT
CTACTCAAATGGATTTCGTGTAGCCCATGCTGACTCAGCGTTACAATTAGTCTATATTCCA

      370     380     390     400     410     420
ATAAGTTGTOTTTACTGAACCGCAAGTTTCTAATTTCCGGTTATGTGTGATAGAGGAAAGT
TATTCAACACAAATGACTTGGCTTCAAAGATTAAAGCCAATACACAGCTATCTCCTTTCA

      430     440     450     460     470     480
GTCTGAAACCTCTAGTACAAAGAAAGGTAAGTTATGTTTGTGGACTTATCTGTTATCACC
CAGACTTTGGAGATCATGTTCTTTCCATTCAATACCAACACCTGAATAGACAATAGTGG

      490     500     510     520     530     540
ACATTTGTACAATCTGTAGGAGAACCCTATGGGAACGAAACGAAAGCGATGCCGAGAATCT
TGTAACATGTTAGACATCCTCTTGATACCCCTTGCTTTGCTTTGCTACGGCTCTTAGA

      550     560     570     580     590     600
CAATTTACCAAGACTTAACACTAACTGGGGATACCCCTAAACAAGAAATGCCTAATAGAAAG
CTTAAATGGTTCTGAATTTGATTGACCCCTATGGGATTGTCTTACGGATTATCTTTTC

      610     620     630     640     650     660
GAGGAAAAAGGCTATAGCACTAGAGCTTGAAAAATCTTGCAAGGGTACGGAGTACTCGTAG
CTCCTTTTCCGATATCGTGATCTCGAACTTTTAGAACGTTCCCATGCCCTCATGAGCATC

      670     680     690     700     710     720
TAGTCTGAGAAAGGTAACGCCCTTTACATGGCAAAGGGGTACAGTTATTGTGTACTAAAA
ATCAGACTCTTCCCATTTGCGGGAAATGTACCGTTTCCCATGTCAATAACACATGATTTT

      730     740     750     760     770     780
TTAAAAATTGATTAGGGAGGAAAAACCTCAAAATGAAACCAACAATGGCAATTTTAGAAAG
AATTTTAACTAATCCCTCCTTTTGGAGTTTACTTTGGTTGTTACCGTTAAAAATCTTTTC

      790     800     810     820     830     840
AATCAGTAAAAATTCACAAGAAAAATATAGACGAAGTTTTTACAAGACTTTATCGTTATCT
TTAGTCATTTTAAAGTGTCTTTTATATCTGCTTCAAAAATGTTCTGAAATAGCAATAGA

      850     860     870     880     890     900

```

LtrB.E1

LtrB.I

EBS 2

EBS 1

LtrA ORF

FIGURE 10

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LtrB-I1.H3.fragment

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TTTACGTCCAGATATTATTACGTGGCGTATCAAAATTTATATTCCAATAAAGGAGCTTC
AAATGCAGGTCTATAAATAATGCACCGCATAGTTTAAATATAAGGTTATTTCTCGAAG

910 920 930 940 950 960
CACAAAAGQAATATTAGATGATACAGCGGATGGCTTTAGTGAAGAAAAAATAAAAAAGAT
GTGTTTTCCTTATAATCTACTATGTCCCTACCGAAATCACTTCTTTTATTTTCTA

970 980 990 1000 1010 1020
TATTCAATCTTTAAAGACGGAACTTACTATCCTCAACCTGTACGAAGAATGTATATTGC
ATAAGTTAGAAATTTTCTGCCCTGAATGATAGGAGTTGGACATGCTTCTTACATATAACG

1030 1040 1050 1060 1070 1080
AAAAAAGAATTCATAAAGATGAGACCTTTAGGAATCCAACTTTCACAGATAAATTGAT
TTTTTCTTAAGATTTTCTACTCTGAAAATCCTTAAGGTTGAAAGTGCTATTTAACATA

1090 1100 1110 1120 1130 1140
CCAAGAAGCTGTGAGAATAATCTTGAATCTATCTATGAACCGGTATTGGAAGATGTGTC
GGTTCCTCGACACTCTTATTAAGAACTTAGATAGATACTTGGCCATAAGCTTCTACACAG

1150 1160 1170 1180 1190 1200
TCACGGTTTGTAGACCTCAACGAAGCTGTACACAGCTTTGAAAACAATCAAAAGAGAGTT
AGTGCCAAAATCTGGAGTTGCTTCGACAGTGTGTCGAAACTTTGTTAGTTTCTCTCAA

1210 1220 1230 1240 1250 1260
TGGCGGCGCAAGATGGTTTGTGGAGGGAGATATAAAGGCTGCTTCGATAATATAGACCA
ACCGCGCGCTTCTACCAAACACCTCCCTCTATATTTCCGACGAAGCTATTATATCTGGT

1270 1280 1290 1300 1310 1320
CGTTACACTCATTGGACTCATCAATCTTAAATCAAGATATGAAAATGAGCCAATTGAT
GCAATGTGAGTAACCTGAGTAGTTAGAATTTTAGTTTCTATACTTTTACTCGGTTAACTA

1330 1340 1350 1360 1370 1380
TTATAAATTTCTAAAAGCAGGTTATCTGGAAAACCTGGCAGTATCACAAAACCTTACAGCGG
AATATTAAAGATTTTCGTCCAATAGACCTTTTGACCGTCATAGTGTTTGAATGTGCGC

1390 1400 1410 1420 1430 1440
AACACCTCAAGGTGGAATTCATCTCTCTTTTGGCCAAACATCTATCTTCATGAATTGGA
TTGTGGAGTTCCACCTTAAGATAGAGGAGAAAACCGGTTGTAGATAGAAGTACTTAACCT

1450 1460 1470 1480 1490 1500
TAAGTTTGTTTTACAACTCAAAATGAAGTTTGACCGAGAAAAGTCCAGAAAGAATAACACC
ATTCAAAACAAAATGTGAGTTTACTTCAAACTGGCTCTTTCAGGTCTTTCTTATTGTGG

1510 1520 1530 1540 1550 1560
TGAATATCGGGAACCTTCACAAATGAGATAAAAAGAATTTCTCACCGTCTCAAGAAGTTGGA
ACTTATAGCCCTTGAAGTGTACTCTATTTTCTTAAAGAGTGGCAGAGTTCTTCAACCT

1570 1580 1590 1600 1610 1620
GGGTGAAGAAAAAGCTAAAGTTCTTTTAGAATATCAAGAAAAACGTAAGATTACCCAC
CCCACTTCTTTTTCGATTTCAAGAAAATCTTATAGTTCTTTTGCATTTTCTAATGGGTG

1630 1640 1650 1660 1670 1680
ACTCCCTGTACCTCACAGACAAATAAAGTATTAAGTATACGTCCGGTATGCGGACGACTT
TGAGGGGACATGGAGTGTCTGTTATTTCATAACTTTATGCAGGCCATACGCCTGCTGAA

1690 1700 1710 1720 1730 1740
CATTATCTCTGTTAAAGGAAGCAAAGAGGACTGTCAATGGATAAAAAGAACAATTAAGT

FIGURE 10 continued

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LtrB-II.X3.fragment

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GTAATAGAGACAATTTCCTTCGTTTCTCCTGACAGTTACCTATTTCTTGTTAATTTTGA

1750	1760	1770	1780	1790	1800
TTTTATTTCATAACAAGCTAAAAATGGAATTGAGTGAAGAAAAACACTCATCACACATAG					
AAAATAAGTATTGTTGCGATTTTACCTTAACTCACCTCTTTTTTGTGAGTAGTGTGTATC					

1810	1820	1830	1840	1850	1860
CAGTCAACCCGCTCGTTTTCTGGGATATGATATACGAGTAAGGAGAAGTGGAAACGATAAA					
GTCAGTTGGGCGAGCAAAAGACCTATACCTATATGCTCATTCCTCTTCACCTTGCTATTT					

1870	1880	1890	1900	1910	1920
ACGATCTGGTAAAGTCAAAAAGAGAACACTCAATGGGAGTGTAGAACTCCTTATTCCTCT					
TGCTAGACCATTTCACTTTTCTCTTGTGAGTTACCTTCACATCTTGAGGAATAAGGAGA					

1930	1940	1950	1960	1970	1980
TCAAGACAAAATTCTGTCATTTATTTTGCACAAGAAAATAGCTATCCAAAAGAAAGATAG					
AGTTCTGTTTAAAGCAGTTAAATAAAAACCTGTTCTTTTATCGATAGGTTTTCTTTCTATC					

1990	2000	2010	2020	2030	2040
CTCATGGTTTCCAGTTTCACAGGAAATATCTTATTCGTTCAACAGACTTAGAAATCATCAC					
GAGTACCAAAGGTCAAGTGTCTTTATAGAATAAGCAAGTTGTCTGAATCTTTAGTAGTG					

2050	2060	2070	2080	2090	2100
AATTTATAATTCTGAATTAAGAGGGATTTGTAATTACTACGGTCTAGCAAGTAATTTTAA					
TAAATATTAAAGACTTAATTTCCCTAAACATTAATGATGCCAGATCGTTTCAATAAAATT					

2110	2120	2130	2140	2150	2160
CCAGCTCAATTATTTTGCTTATCTTATGGAATACAGCTGTCTAAAAACGATAGCCTCCAA					
GGTCGAGTTAATAAACGAATAGAATACCTTATGTCGACAGATTTTGTCTATCGGAGGTT					

2170	2180	2190	2200	2210	2220
ACATAAGGGAAACACTTTCAAAAACCATTTCCATGTTTAAAGATGGAAGTGGTTCTGTTGGG					
TGTATTCCTTGTGAAAGTTTTTGGTAAAGGTACAAATTTCTACCTTCACCAAGCACCCC					

2230	2240	2250	2260	2270	2280
CATCCCGTATGAGATAAAGCAAGGTAAAGCAGCGCCGTTATTTTGCAAATTTTAGTGAATG					
GTAGGGCATACTCTATTTCTGTTCCATTCGTGCGGGCAATAAACGTTTAAATCACTTAC					

2290	2300	2310	2320	2330	2340
TAAATCCCCTTATCAATTTACGGATGAGATAAGTCAAGCTCCTGTATTGTATGGCTATGC					
ATTTACCCGAATAGTTAAATGCCTACTCTATTCAATTCGAGGACATAACATACCGATACG					

2350	2360	2370	2380	2390	2400
CCCGAATACTCTTGAAAACAGGTTAAAAGCTAAATGTTGTGAATTATGTGGAACATCTGA					
GGCCTTATGAGAACTTTGTCCAATTTTCGATTTACAACACTTAATACACCTTGTAAGT					

2410	2420	2430	2440	2450	2460
TGAAAATACTTCCTATGAAATTCACCATGTCAATAAGGTCAAAAATCTTAAAGGCAAGA					
ACTTTTATGAAGGATACTTTAAGTGGTACAGTTATTCCAGTTTTTAGAATTTCCGTTTCT					

2470	2480	2490	2500	2510	2520
AAAATGGGAAATGGCAATGATAGCGAAACAACGTAACCTCTTGTGTATGCTTTTCATTG					
TTTTACCCCTTACCGTTACTATCGCTTGTGTCATTTTGAGAACACATACGAAAGTAAC					

2530	2540	2550	2560	2570	2580
TCATCGTCACGTGATTTCATAAACACAACTGAATTTTACGAAACGAACAAATAACAGAGCCG					
AGTAGCAGTGCACTAAGTATTGTGTTCACTTAAAAATGCTTGCTTGTATTGTCTCGG					

FIGURE 10 continued

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LtrB-11.E3.fragment

Monday, February 23, 1998 2:44 PM

2590 2600 2610 2620 2630 2640
TATACTCCGAGAGGGGTACGTACGGTTCCTCGAAGAGGGTGGTGCAAACCAAGTCACAGTAA
ATATGAGGCTCTCCCATGTCATGCCAAGGGCTTCTCCACCAACGTTGGTCAGTGTCAAT

LtrB I

2650 2660 2670 2680 2690 2700
TGTAACAAGGCGGTACCTCCCTACTTCACCATATCATTTTAAATCTACGAATCTTTAT
ACACTTGTTCCTCCCATGAGGGATGAAGTGTATAGTAAAAATTAAGATGCTTAGAATA

LtrB.E2

2710 2720 2730 2740 2750 2760
ACTGGCAAAACAATTTGACTGGAAAGTCATTCTAAAGAGAAAAACAAAAGCGGCAAGCT
TGACCGTTGTAAACTGACCTTTCAGTAAGGATTCTCTTTTGTTTTCGCCGTTTCGA

T
A

FIGURE 10 continued

INTERNATIONAL SEARCH REPORT

 International application No.
 PCT/US98/03990
A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12Q 1/68

US CL : 435/6

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 91.1, 91.3, 91.31, 91.51

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Searched inventors and keywords: integrase or RNP or group II intron and methods cleaving in APS, CAPLUS, MEDLINE
SCISEARCH, WPIDS, BIOSIS.**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P — A, P	US 5,698,421 A (LAMBOWITZ et al) 16 December 1997, see entire document.	1, 2, 4, 13-19, 24 3, 5-12, 20-23
A	US 5,498,531 A (JARRELL) 12 March 1996, see columns 8-11, columns 25-28 and columns 30-31.	1-24
A	ZIMMERLY et al. Group II Intron Mobility Occurs by Target DNA-Primed Reverse Transcription. Cell. August 1995, Vol. 82, No. 4, pages 545-554, see entire document.	1-24
A	KENNEL et al. Reverse Transcriptase Activity Associated with Maturase-Encoding Group II Introns in Yeast Mitochondria. Cell. April 1993, Vol. 73, No. 1, pages 133-146, see pages 133-135.	1-24



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	* T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
* A		document defining the general state of the art which is not considered to be of particular relevance
* B		earlier document published on or after the international filing date
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* P		document published prior to the international filing date but later than the priority date claimed
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	* A	document member of the same patent family

Date of the actual completion of the international search

05 JUNE 1998

Date of mailing of the international search report

23 JUN 1998

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